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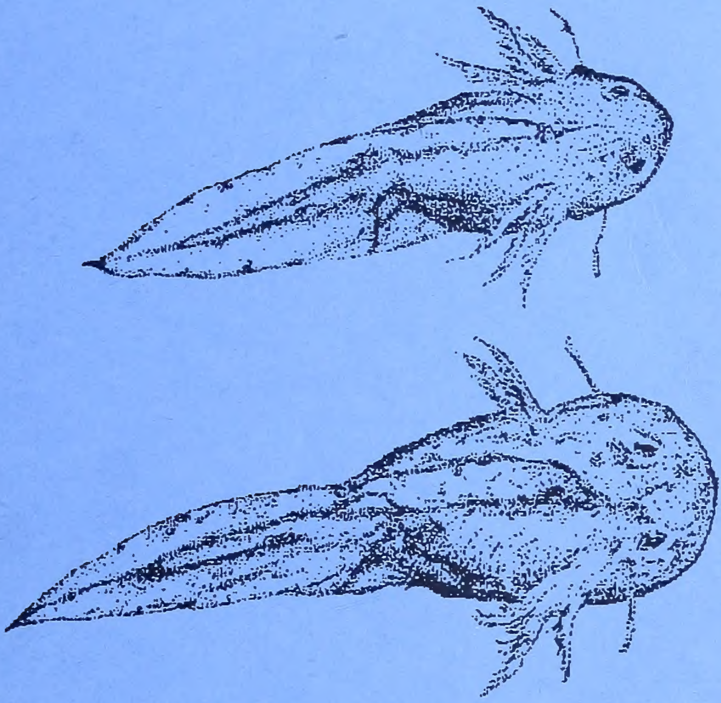

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Cover Illustration: Large- and small-headed larvae of *Hynobius retardatus*. Redrawn from figures by Yukihiro Kohmatsu

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Effects of Head Shape Variation on Diet Selection in Larvae of the Salamander *Hynobius retardatus*

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Abstract: Using stomach content analysis, I assessed effects of head morphology of larval salamanders (*Hynobius retardatus*) on the selectivity of diets in nature. Large-headed morphs of the larvae chiefly ate terrestrial invertebrates, whereas the most abundant prey item of small-headed morphs was Diptera larvae. This suggests that larvae of *H. retardatus* change their prey in association with the head shape. I thus consider that the flexible development of a larger head induced by larval density of this species would change their diet selection.

Key words: Diet selection; Head shape; *Hynobius retardatus*; Salamander; Stomach contents

INTRODUCTION

Trophic polymorphism, in which different morphs often exhibit different uses of food resources even in sympatric conditions, has been reported in many animals (Malmquist, 1992; Schluter, 1995; Skulasson and Smith, 1995). Such feeding specialization among morphs is thought to provide organisms with potentials for intraspecific niche divergence (Rosenzweig, 1978).

Several salamanders have a dimorphic life history, consisting of cannibalistic and typical morphs in the larval period (see review in Crump, 1992). *Hynobius retardatus*, a hynobiid salamander which breeds in small, temporary ponds in northern Japan (Sato, 1993), is also reported to have flexible development in larval head size (Wakahara, 1995), with large-headed morph larvae being characterized by a wide head and mouth, and relatively round snout (Fig. 1). Since prey items of larvae of *H. retardatus* are limited by their gape size (Ohdachi, 1994), the large-headed morphs are expected to feed on larger prey organisms than the small-headed morphs.

In fact, the cannibalistic morph larvae of the tiger salamander (*Ambystoma tigrinum*), characterized by relatively broad heads, are known to feed on animals including conspecific larvae that are larger than prey of the typical morph larvae of this species (Collins and Holomuzki, 1984). Moreover, a previous study on *A. tigrinum* predicted that the shift of prey selectivity of larvae involved in changes in head morphology influences the dynamics of the community of a pond where they occur

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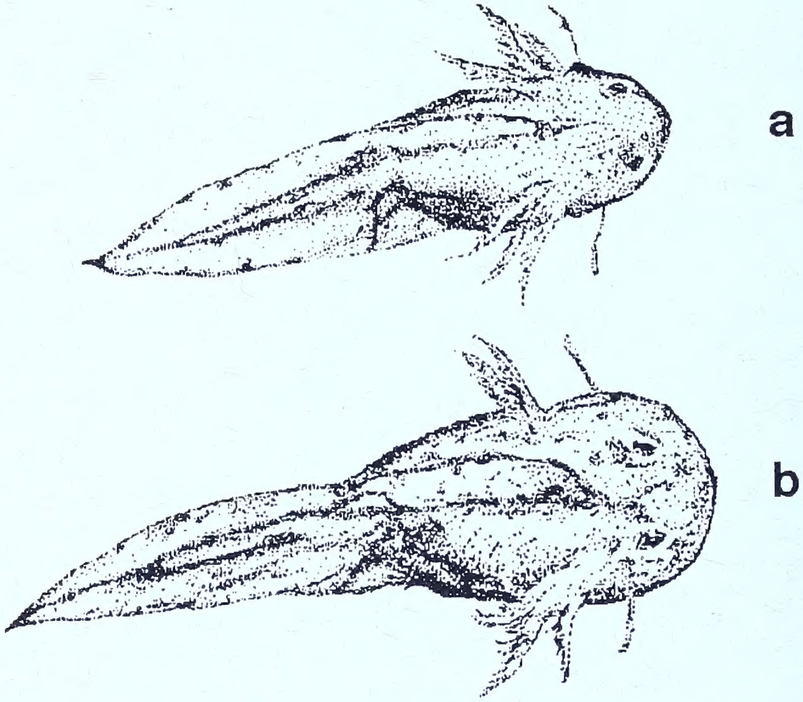

FIG. 1. Representatives of large- (a) and small-headed larvae (b) of *Hynobius retardatus* (redrawn from photographs).

(Holomuzki et al., 1994). With respect to *H. retardatus* larvae, however, no studies have been performed on the effects of head shape on their diet selectivity.

I compared the stomach contents between large-headed and small-headed larvae of *H. retardatus* collected from a natural pond. The purpose of this study was to examine the effect of larval head shape on diet selection in this species.

MATERIALS AND METHODS

A total of 44 larvae of *H. retardatus* were sampled on 3 August 1998 from a pond (330 m^2 surface area, 75 cm deep) in Tomakomai Experimental Forest of Hokkaido University (TOEF; 42°37'N, 141°20'E), southwestern Hokkaido, Japan. The species lays eggs in the pond from late April to early May and metamorphoses in September. I captured larvae at the middle stage (i.e., digital differentiation I–VI; stage 48–53 of Iwasawa and Yamashita, 1991). The sampled larvae were immediately sacrificed with tricane methane sulfonate (MS-222), and were measured for snout-vent length (SVL, distance from anterior tip of snout to posterior margin of vent) and head width (HW, width of head at the broadest portion) with a digital caliper to the nearest 0.1 mm. Their stomach contents were then examined by abdominal dissection under a binocular microscope. Each food item was first categorized as salamander larvae, detritus, and terrestrial and aquatic invertebrates. Items categorized as invertebrates were further identified to the order level (Appendix). The wet mass of items composing each dietary category was weighed to the nearest 0.01 mg after blotting.

The head shape of larval *H. retardatus* shows continuous variation within a population (Nishihara, 1996). To assess the head shape of larvae, residuals of a simple linear regression of HW on SVL were examined. The HW of larvae was significantly correlated with SVL (r_2=0.899, $p<0.001$: for $\log_{10}$ transformed data). Each larva was then classified as either large- or small-headed according to the residuals of HW on SVL (plus or minus value, respectively), although there was no significant difference in SVL or HW between large- and small-headed larvae (tested a posteriori: SVL, t_{42}=0.149, $p>0.882$; HW, t_{42}=1.567, $p>0.125$, both for $\log_{10}$ transformed data). Wet mass of total diets of larvae was compared between large- and small-headed groups using Student's t-test. The proportional contribution of each prey category to the total stomach contents was determined for each head shape group. For this analysis, terrestrial invertebrates were pooled together irrespective of their taxonomic allocations due to the small sample size for each invertebrate order. Overall diet composition by wet mass was compared between the two groups using a MANOVA with individuals as replicates. Subsequently, the mean proportion of each dietary category was separately analyzed by ANOVA. In order to standardize variances for all statistical tests, all absolute values and proportions were $\log_{10}$ transformed and arcsine-square-root transformed, respectively.

RESULTS

Total wet mass of large-headed larvae was significantly larger than that of small-headed larvae (t=3.77, $p<0.001$). One of the small-headed larvae had an empty stomach. The overall dietary composition of large- and small-headed larvae differed significantly (Wilks' lambda=0.51, F=5.05, $p<0.001$ by MANOVA; Table 1). A large proportion of the diet of the large-headed larvae comprised terrestrial invertebrates, whereas for the small-headed larvae, detritus (fine litter remains) and Diptera larvae were the main stomach contents, with terrestrial invertebrates composing no more than 2% of the diet (Fig. 2). Separate ANOVAs showed significant differences in the percentage of terrestrial invertebrates and Diptera larvae between the two groups of larvae, but not in the remaining dietary categories (Table 1).

DISCUSSION

The results of the stomach content analysis showed that the prey items of larval *H. retardatus* vary in accordance with variation in head morphology (Fig. 2). The large-headed larvae ate larger prey items than the small-headed larvae (Appendix). In larval *H. retardatus*, possession of a wide head and mouth is advantageous in that it permits the larva to feed on larger prey (Ohdachi, 1994). Nevertheless, the

TABLE 1. Results of ANOVAs for diet proportion of *Hynobius retardatus*.

Source	df	MS	F	p*
Terrestrial invertebrates	1, 42	1.2865	17.036	0.0002
Diptera larvae	1, 42	0.932	0.9077	0.0044
Ephemeroptera	1, 42	0.0057	0.9112	0.3453
Trichoptera	1, 42	0.01	1.40195	0.2004
Copepoda (Cyclopoida)	1, 42	0.0005	0.0071	0.9334
Salamander	1, 42	0.026	0.5307	0.0925
Detritus	1, 42	0.9648	6.58	0.014

* Reduced alpha level=0.007 (i.e., 0.005/7)

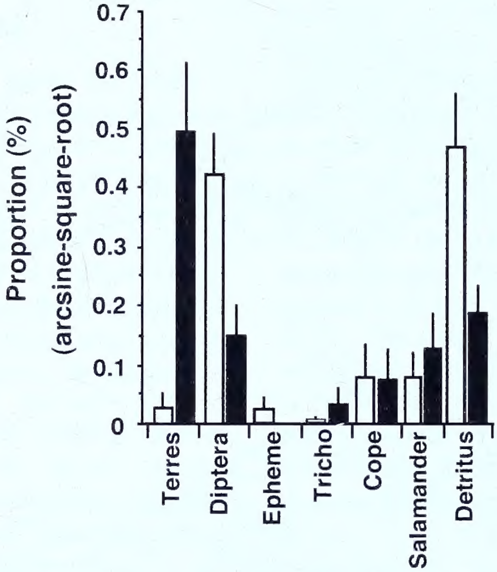

FIG. 2. Proportion in wet mass (mean + SE) of diet composition in large- (■; n=21) and small-headed larvae (□; n=23) of *Hynobius retardatus* collected from a pond in Tomakomai Experimental Forest. Terres=terrestrial invertebrates; Diptera=Diptera larvae; Epheme=Ephemeroptera nymphs; Tricho=Trichoptera larvae; Cope=Copepoda; Salamander=salamander larvae (*H. retardatus*); Detritus=fine litter.

absolute value of HW did not differ between the two groups of larvae. It is therefore likely that the variation in diet was caused not by the difference in gape size between the large- and small-headed larvae, but by other factors. Considering the difference in shape of the snout tip between the two groups (i.e., relatively pointed in the small-headed larvae, and more rounded in the large-headed larvae: Fig. 1), it is probable that the dietary variation actually reflects difference in foraging environments: the small-headed larvae may forage using their snout for digging around the pond bottom where their main food items, detritus and Diptera larvae, chiefly occur, whereas the large-headed larvae may chiefly feed on fallen terrestrial invertebrates near the water surface.

For larvae of some salamanders, variation in diet is a cause of variation in growth in body mass (Brunkow and Collins, 1996). The present results suggest that *H. retardatus* larvae also shift prey items under the influence of flexible development of large head width (Fig. 2). Such an intra- populational variation in diet could influence the population dynamics. For instance, the divergence of diet selection within a population may reduce intraspecific competition, thereby allowing a greater population density and higher averages of individual fitness (Polis, 1984). Because individual variation in head shape depends on the initial density (Kohmatsu et al., 2001), the prediction of population regulation for *H. retardatus* should include information on such population demography.

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APPENDIX

Size of food items (mean, followed by SE in parentheses) of larval *Hynobius retardatus*.

Food item	Wet weight (mg)	Body length (mm)
Terrestrial invertebrates		
Araneae	23.20	5.50
Collembola	0.36 (0.02)	0.94 (0.04)
Lepidoptera	23.42 (3.79)	6.30 (0.37)
Coleoptera	30.10 (4.40)	5.50 (1.00)
Dermaptera	11.20	3.50
Hymenoptera	0.51 (0.02)	2.94 (0.06)
Isopoda	7.62 (2.30)	2.58 (0.74)
Diptera	0.58 (0.15)	2.38 (0.24)
Aquatic invertebrates		
Diptera larvae	0.27 (0.03)	2.70 (0.34)
Ephemeroptera	0.16 (0.02)	3.60 (0.33)
Trichoptera	6.33 (1.86)	4.50 (0.76)
Cyclopoida	0.10	0.57 (0.09)

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Genetic Relationships of a Morphologically Unique Population of the Genus *Eumeces* (Scincidae: Squamata) from Iotorishima Island, Ryukyu Archipelago, as Revealed by Allozyme Data

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Abstract: The Ryukyu five-lined skink, *Eumeces marginatus*, is an endemic species of the central and northern Ryukyus, and is composed of two morphologically defined subspecies, *E. m. marginatus* from the Okinawa Group, and *E. m. oshimensis* from the Amami and Tokara Groups. A population of the genus *Eumeces*, recently found on Iotorishima Island (an uninhabited islet located north of the main island chain of the Ryukyus), resembles *E. m. oshimensis* in having a relatively short median white line on the tail, but is distinct from any population of *E. marginatus* in having more midbody scale rows. To assess the taxonomic status of the Iotorishima population from a historical viewpoint, we examined its genetic relationships with *E. marginatus* populations. The degree of genetic differentiation between the Iotorishima population and a population of *E. m. oshimensis* from Tokunoshima, northern Amami islands, was fairly low (D=0.043). Cluster analysis of genetic data also indicated that the Iotorishima population constitutes one of the two major clusters within *E. marginatus* together with four *E. m. oshimensis* populations from the northern Amami and southern Tokara islands. Thus, it is likely that the number of midbody scale rows in the Iotorishima population has increased rapidly after its relatively recent isolation. Taxonomically, the Iotorishima population seems to be most appropriately assigned to *E. m. oshimensis*.

Key words: *Eumeces marginatus*; Reptilia; Allozyme: Iotorishima Island; Ryukyu Archipelago

INTRODUCTION

Eumeces marginatus (Hallowell, 1861) is a moderate-sized scincine lizard endemic to the Ryukyu Archipelago. The range of this species includes most islands of the Okinawa and Amami Groups, and

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Takarajima, Kodakarajima and Kojima Islands of the Tokara Group (Toyama, 1989; Ota et al., 1994). Two subspecies, *E. m. marginatus* from the Okinawa Group, and *E. m. oshimensis* from the Amami and Tokara Groups, are generally recognized on the basis of scutellation and coloration (e.g., Nakamura and Uéno, 1963). Although some external characters, such as the scale row number and juvenile tail coloration, are known to vary among island populations of *E. marginatus*, variations are not consistent with the geographic arrangement of populations and thus are considered to have little significance in terms of their historical relationships (Toyama, 1989). In fact, Kato et al. (1994), in the phylogenetic analyses of allozyme data for 19 populations representing six species of the East Asian *Eumeces*, recognized two assemblages of island populations within *E. m. oshimensis*, of which one is genetically closer to *E. m. marginatus* than to the other "consubspecific" assemblage.

Recently, Toyama (1995) reported on two *Eumeces* specimens collected for the first time from Iotorishima Island, an uninhabited islet 65 km west of Tokunoshima Island, the Amami Group (Fig. 1). These specimens, while most resembling *E. m. oshimensis* in head scutellation and coloration, differed from *E. marginatus* in having more rows of scales around the midbody (34, vs 24–30; Toyama, 1995).

In May 1997, four additional specimens of *Eumeces* were collected from Iotorishima Island by S. Ikehara and colleagues during a comprehensive scientific survey of the island. We sampled fresh tissues from

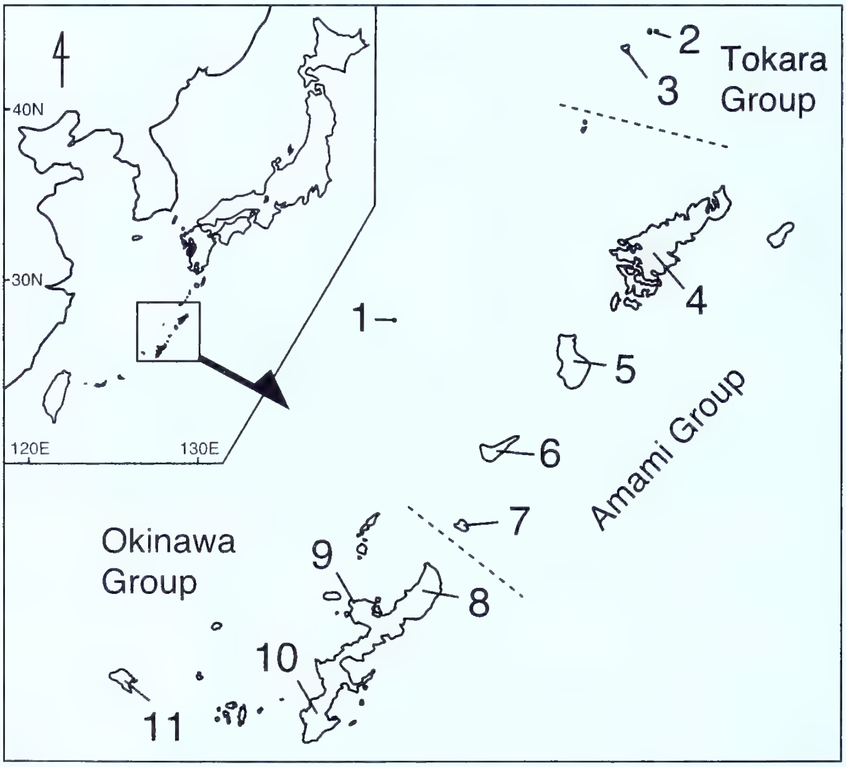

FIG. 1. A map of the central Ryukyus, showing sampling localities of *Eumeces marginatus*. 1=Iotorishima, 2=Kodakarajima, 3=Takarajima, 4=Amamioshima, 5=Tokunoshima, 6=Okinoerabujima, 7=Yoronjima, 8=Yona, 9=Bise, 10=Shuri, 11=Kumejima.

these specimens, and examined them electrophoretically together with tissue samples of *E. marginatus* from almost whole of its range. Our purpose was to determine the taxonomic status of the Iotorishima population on the basis of its relationships with *E. marginatus* populations reflected by allozyme variations.

MATERIALS AND METHODS

We used four specimens from Iotorishima Island and 224 *Eumeces marginatus* specimens from eight islands of the Ryukyu Archipelago: the Tokara Group (Takarajima and Kodakarajima), the Amami Group (Amamioshima, Tokunoshima, Okinoerabujima, and Yoronjima), and the Okinawa Group (Okinawajima and Kumejima) (Fig. 1). All specimens from the same island, except for those from Okinawajima, were combined for the analyses under an a priori assumption that they represent a single randomly breeding population. Specimens from three localities within Okinawajima were treated as three separate samples, because the island's large size (120 km in length, 10 km in breadth and 1200 km^2 in area) may have enhanced subdivisions of its lizard population.

Homogenized tissue extracts were analyzed by horizontal starch gel electrophoresis. Sample preparation and electrophoretic procedures followed Shaw and Prasad (1970) and Harris and Hopkinson (1976) as modified by Kato et al. (1994). Enzymes examined and buffer systems used here were almost same as those in Kato et al. (1994). Nevertheless, Ah-2 (Kato et al., 1994) was excluded from the analyses, because products of this locus were not consistently clear enough to permit reliable scoring. Instead, peptidase was examined for the first time for the Japanese *Eumeces*, and resultant bands from liver extracts were interpreted as being controlled by a single locus, Pep-D. A total of 22 loci of 16 enzymes (i.e., Aat-1, Aat-2, Ada-2, Ah-1, Ck-2, Est-4, Fh, Gdh, Gpi, Idh-1, Idh-2, Ldh-1, Ldh-2, Ldh-3, Mdh-1, Mdh-2, Mpi, Pep-D, 6Pgdh-1, Pgm-1, Pgm-2, and Sod-1) were thus examined.

Allozyme data were treated as products of codominant alleles at a given locus. For some specimens of *E. marginatus*, allozyme data, taken from our previous study (Kato et al., 1994), were subjected to the analyses. All loci were evaluated for genetic polymorphism (mean number of alleles per locus, proportion of polymorphic loci, and observed mean heterozygosity). In order to estimate overall genetic differentiation among populations, Nei's (1978) unbiased genetic distance (D) was calculated on the basis of population allele frequencies. The resultant pairwise D matrix was further subjected to analyses by the UPGMA algorithm (Sneath and Sokal, 1973) for the purpose of estimating genetic relationships among populations. All computations were carried out using the BIOSYS-1 computer program (Swofford and Selander, 1981).

In addition, we quantified a few external characters (snout–vent length, tail length, extent of the median white line on tail, and the number of midbody scale rows), and also investigated the postnasal condition for the Iotorishima specimens.

RESULTS

Of the 22 presumptive loci analyzed, nine (Aat-2, Ck-2, Fh, Gdh, Ldh-1, Ldh-2, Mpi, Pgm-1 and Sod-1) were fixed in all samples. Allele frequencies at the other 13 polymorphic loci are given in Table 1. At six of these (Idh-1, Idh-2, Ldh-3, Mdh-1, Mdh-2, and Pgm-2), the predominant (frequency of occurrence >50%) electromorph was identical in all samples. The Iotorishima sample had alleles common to the Tokunoshima sample at all loci, and all but the Est-4 locus were fixed or nearly fixed for these shared alleles. With respect to the latter locus, all of the Iotorishima specimens had a homozygote of an allele

TABLE 1. Allele frequencies at 13 polymorphic loci and variability estimates for 11 popoulations of *Eumeces marginatus*. Locality numbers correspond to those in Fig. 1. A=mean number of alleles per locus, P=percentage of polymorphic loci, *H*=observed mean heterozygosity.

	Population (N)										
Locus	1 (4)	2 (18)	3 (12)	4 (19)	5 (21)	6 (22)	7 (22)	8 (30)	9 (31)	10 (24)	11 (25)
Aat-1											
a						.886	.952		.050	.021	
b	1.000	1.000	1.000	1.000	1.000	.023			.100	.109	
c						.091	.048	1.000	.850	.870	.320
d											.680
Ada											
a	1.000	1.000	.958	1.000	1.000						
b			.042					.917	.155	.773	1.000
c						1.000	1.000	.083	.845	.227	
Ah-1											
a	1.000			.053	1.000	.955	.977	1.000	1.000	.861	1.000
b		1.000	.958	.921		.045	.023			.139	
c			.042	.026							
Est-4											
a		.028			.025			.038			
b		.972	1.000	1.000	.950	.929	.955				
c	1.000				.025	.071	.045	.924	.100	.409	.100
d								.038	.900	.591	.900
Gpi											
a	1.000				1.000	.045					
b		1.000	1.000	1.000		.955	1.000	1.000	1.000	1.000	1.000
Idh-1											
a								.017			
b	1.000	1.000	1.000	1.000	.976	1.000	1.000	.983	1.000	1.000	1.000
c					.024						
Idh-2											
a										.159	
b	1.000	1.000	1.000	1.000	1.000	1.000	1.000	1.000	1.000	.841	1.000
Ldh-3											
a										.022	.020
b	1.000	1.000	1.000	1.000	1.000	1.000	1.000	1.000	1.000	.978	.980
Mdh-1											
a	1.000	1.000	1.000	1.000	1.000	1.000	1.000	1.000	1.000	1.000	.960
b											.040
Mdh-2											
a								.086			
b	1.000	1.000	1.000	1.000	1.000	1.000	1.000	.880	1.000	1.000	1.000
c								.034			

TABLE 1. Extended

	Population (N)										
Locus	1 (4)	2 (18)	3 (12)	4 (19)	5 (21)	6 (22)	7 (22)	8 (30)	9 (31)	10 (24)	11 (25)
Pep-D											
a								.786	.016		
b	1.000	1.000	1.000	1.000	1.000	1.000	.969	.214	.984	1.000	1.000
c							.031				
6Pgdh-1											
a			.083	.059							
b	1.000	1.000	.917	.941	1.000	.182	.091	1.000	1.000	.556	1.000
c						.818	.909				
d										.444	
Pgm-2											
a							.023		.016		.040
b	1.000	1.000	1.000	1.000	1.000	1.000	.977	.857	.887	1.000	.960
c								.143	.097		
A	1.0	1.0	1.1	1.1	1.1	1.3	1.3	1.4	1.3	1.4	1.2
(SE)	(0.1)	(0.0)	(0.1)	(0.1)	(0.1)	(0.1)	(0.1)	(0.1)	(0.1)	(0.1)	(0.0)
P	0.0	4.5	13.6	9.1	9.1	22.7	27.3	27.3	22.7	31.8	22.7
H	0.000	0.003	0.015	0.002	0.007	0.033	0.015	0.019	0.033	0.034	0.033
(SE)	(0.000)	(0.003)	(0.009)	(0.002)	(0.005)	(0.016)	(0.007)	(0.011)	(0.016)	(0.013)	(0.022)

TABLE 2. Matrix of genetic distance (Nei, 1978) between 11 populations of *Eumeces marginatus*. A negative value set to -0.000.

Population	1	2	3	4	5	6	7	8	9	10
1. Iotorishima	—									
2. Kodakarajima	0.145	—								
3. Takarajima	0.146	-0.000	—							
4. Amamioshima	0.141	0.000	-0.000	—						
5. Tokunoshima	0.043	0.096	0.094	0.090	—					
6. Okinoerabujima	0.223	0.174	0.167	0.166	0.175	—				
7. Yoronjima	0.243	0.188	0.181	0.180	0.191	0.000	—			
8. Yona	0.182	0.233	0.230	0.229	0.231	0.196	0.210	—		
9. Bise	0.174	0.179	0.177	0.174	0.178	0.108	0.122	0.095	—	
10. Shuri	0.162	0.168	0.163	0.163	0.183	0.126	0.139	0.059	0.034	—
11. Kumejima	0.182	0.186	0.182	0.181	0.185	0.161	0.174	0.090	0.053	0.036

which occurred in very low frequency in the Tokunoshima sample.

In the present samples, values of the mean number of alleles per locus (A), proportion of polymorphic loci (P), and observed mean heterozygosity per locus (H) ranged from 1.0-1.4, 0.0-31.8, and 0.000-0.034, respectively (Table 1). The Iotorishima sample exhibited neither polymorphism nor allelic heterozygosity.

Pairwise comparisons of samples by Nei's (1978) genetic distance coefficient (D)

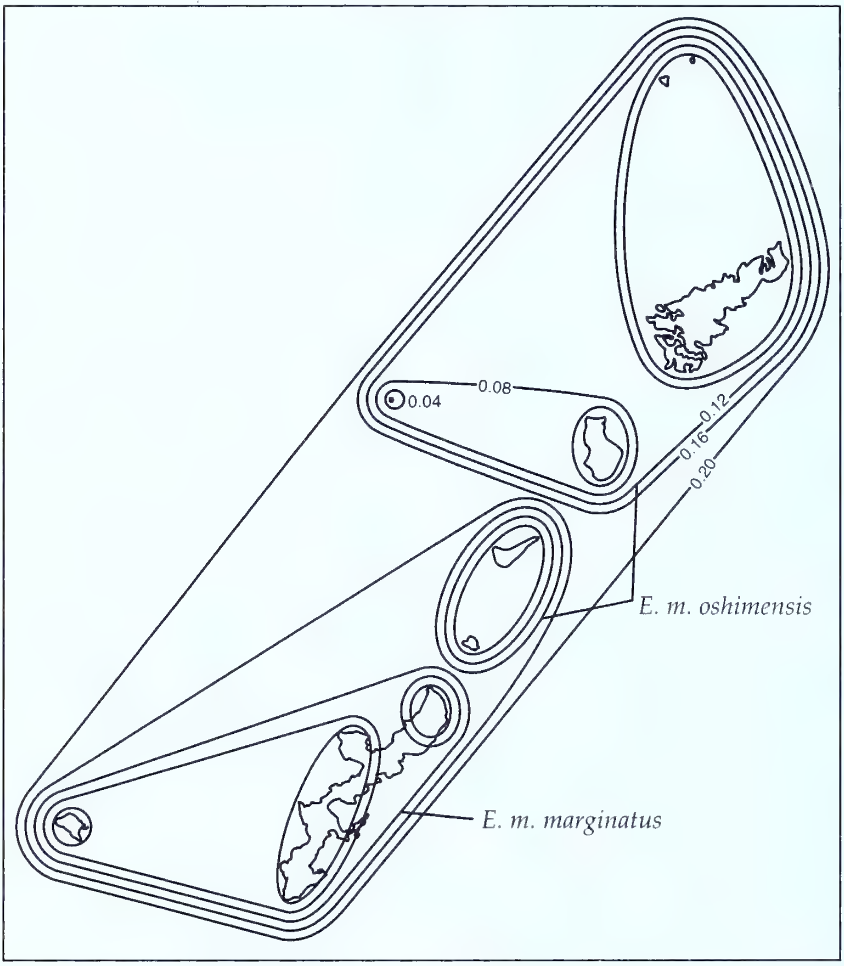

FIG. 2. Genetic relationships among 11 populations of *Eumeces marginatus* based on contours of Nei's (1978) genetic distance values derived from a UPGMA algorithm.

are presented in Table 2. The D value obtained between the Iotorishima and Tokunoshima samples was very small (0.043), whereas those of the former with other *E. marginatus* samples were larger (0.141–0.243).

Figure 2 shows genetic grouping of samples derived from the UPGMA clustering of pairwise D matrix. Populations currently assigned to *E. m. marginatus* were grouped together with high genetic similarities. By contrast, a great genetic heterogeneity was confirmed for *E. m. oshimensis* in the current delimitation, indicating a relatively distinct differentiation between the southern Amami and the northern Amami-southern Tokara assemblages, as reported previously (Kato et al., 1994). In this analysis, the Iotorishima sample was located in the cluster otherwise consisting of the northern Amami-southern Tokara *E. m. oshimensis*.

The four examined specimens from Iotorishima Island invariably had a medial white line not exceeding the proximal one third of the tail and 34 rows of midbody scales, and lacked the postnasal scale

TABLE 3. Measurements (in mm) and scale characters of the Ryukyu five-lined skink, *Eumeces marginatus*, from Iotorishima Island. SVL=snout-vent length, TL=tail length, EL=extent of the median white line on tail, SR=number of midbody scale rows, PN=postnasal condition.

No.	Date of Collection	Sex	SVL	TL	EL	SR	PN
KUZ34568	10 May 1997	female	48.6	89.4	>1/3	34	absent
KUZ34569	10 May 1997	male	51.5	15.2+*	—**	34	absent
KUZ34570	10 May 1997	male	49.3	89.1	>1/3	34	absent
KUZ34571	11 May 1997	female	48.8	87.1	>1/3	34	absent

*: + tail broken.

**: — not clear.

(Table 3). These character states are identical to those in the two previously collected specimens reported by Toyama (1995).

DISCUSSION

Toyama (1995), in the description of external characters in the two previous specimens from Iotorishima Island, stated that their head scutellation seemingly fell within the range of variation in *Eumeces marginatus*. He further noted that in these specimens the extent of the median white line on the tail, a character used to discriminate the two subspecies of *E. marginatus*, was consistent with that of *E. m. oshimensis* rather than that of *E. m. marginatus*. However, he also noted that the specimens had many more rows of midbody scales (34) than *E. marginatus* (24–30). Examination of the four additional specimens confirmed these morphological characteristics for the Iotorishima population of *Eumeces*, and this alone may suggest a genealogical distinctness of this population from *E. marginatus*.

However, results of analyses of allozyme data indicate that the Iotorishima population is genetically remarkably similar to *E. marginatus*, and is much closer to the Tokunoshima population of *E. m. oshimensis* than any other consubspecific populations. This suggests an exclusively close historical affinity of the two populations, and we thus believe that the Iotorishima population should be classified as *E. m. oshimensis*, even though it exhibits a unique state in the number of midbody scale rows. It is probable that in such a small island population as that on Iotorishima Island the midbody scale rows have increased rapidly in response to selection pressure from some environmental factor there.

The Iotorishima population has alleles common to the Tokunoshima population at all loci and the D value between these populations was very small (0.043). Such close genetic resemblance between the Iotorishima and Tokunoshima populations suggests that these populations have been very recently isolated. However, Iotorishima Island is situated 65 km west of Tokunoshima Island, and is separated from the latter, as well as from other islands having *E. marginatus* populations, by straits no less than 500 m in depth (MSAJ, 1978). Since sea level dropped by 120–140 m only during late Pleistocene (Ota et al., 1993), Iotorishima should have remained isolated then. The above result thus suggests a relatively recent overseas dispersal of the lizard between these islands. Otherwise, it necessitates an ad hoc assumption for the formation of a land bridge between them and its recent rapid submergence.

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Phylogenetic Relationships of a Chinese Frog, *Rana zhengi* Zhao, 1999 Inferred from Mitochondrial Cytochrome b Gene Sequences (Amphibia, Ranidae)

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Abstract: In order to assess phylogenetic relationships of a Chinese frog, *Rana zhengi* Zhao, 1999, the sequences of 587 base pairs of the mitochondrial cytochrome b gene are compared with six species of Japanese (*R. pirica*, *R. ornativentris*, *R. japonica*, *R. okinavana*, *R. tagoi*, and *R. tsushimensis*), one species of Taiwanese (*R. sauteri*), one species of Russian (*R. amurensis*), and two species of European brown frogs (*R. temporaria* and *R. dalmatina*). The resultant phylogenetic tree suggests monophyly of *R. zhengi* and these brown frogs, and the earliest divergence of *R. zhengi* among all the ingroup species examined. Monophyly of *R. zhengi* and *R. sauteri* was rejected. For this reason, placement of *R. zhengi* within the brown frogs of the *R. temporaria* group requires further study, and separation of *R. zhengi* and *R. sauteri* as a distinct genus or subgenus *Pseudorana* is not supported.

Key words: Brown frog; Cytochrome b; Phylogeny; *Pseudorana*; *Rana zhengi*; *R. sauteri*; China

INTRODUCTION

Rana zhengi was described from Zhangcun Village, Hongya Co., Sichuan Province, China (Zhao, 1999). Zhao (2000a) considered this species to be related to members of the genus (Fei et al., 1990) or subgenus (Dubois, 1992) *Pseudorana*, which includes *R. sauteri* from Taiwan (Fei et al., 1990). This species, however, has been proven to include cryptic species (Chou and Lin, 1997) and is considered to form a species complex (Tanaka-Ueno et al., 1998a). In our previous study, we showed that two populations of *R. sauteri* (sensu Chou and Lin, 1997), while being distant from each other phylogenetically, form sister groups with some Japanese brown frogs, and that the genus or subgenus *Pseudorana* is invalid (Tanaka-Ueno et al., 1998a).

From these lines of previous information, *R. zhengi* might be a member of the brown frogs of the *R. temporaria* group (Boulenger, 1920), but no studies have ever been

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                    1                                               50                                               100
X. laevis           CAAATCATTACAGGATTATTCTTAGCTATACATTATACAGCAGACACATCTATAGCCTTCTCATCAGTAGCCCATATTTGCCGTGACGTTAACTATGGAT
R. catesbeiana      ······G·C··T··C······C····C·····C········C·····T··CC·T··A·········TA·C··T··C··C·········T·····TA·C··C·
R. zhengi           ······GC···T··CC····TC·C··C·····C·····G··C·····C··CC·T··A·····C··CA·C··T·····C················A·C··C·
R. temporaria       ······GCC··C··C·····T··G········C·········C··T··C··CC·C··A·········TA·C········C··T··G··T·····TA·C··C·
R. dalmatina        ······G····T··CC·····C·T·····G··C··C·····C·····G··CC·C··A··T·····TA·T··T··C··C·····C·········TA····C·
R. sauteri (Sanyi)  ······GCC··C··CC·····C····A·····C··C·····T·····T···C·G··A··T·····TA·C········C·········T·····TA····C·
R. sauteri (Wulai)  ······GCC··C··CC·····C·G··C·····C·····G··T·····C··CC····A··T··C··T··C·····C··C·········T······A·C··C·
R. amurensis        ·····TGC···T···C·····C····C·····C·····G··T··T··T···C·G··A·········TA·C··A··C··C·····A··T·····TA·C····
R. tagoi            ······GCC··C··CC·····C·G········C··C··G··C··T··T··CC·C··A·········TA·C·····C··C·····C··T··A··TA····C·
R. okinavana        ······GCC··C··CC·····C·T··C·····C··C··G··T··T··T··CC····A··T··T··TA·T·····C··C·····C··T··A···A·C····
R. tsushimensis     ······GCC··C··CC·····C·G··C·····C·····G··T·····C··CC····A··T··C··TA·C·····C··C·········T······A·C··C·
R. japonica         ··G···GCC··C···C····TC·G··C·····C··C······T··T··T··CC····A··T·····TA·C········C·····C··T··C···A·C··C·
R. ornativentris    ······GCC··G···C·····C··········C··········T··T··C···C····A··T·····TA·T·····C··C·····A··T··A···A····C·
R. pirica           ······GCC··C··GC····TC····C·····C··········G··T··T···C····G··T·····CA·C·····C··C··T··A··T··A··TA·C··C·

                    101                                            150                                               200
X. laevis           GATTAATTCGCAATCTCCATGCCAATGGAGCCTCATTCTTCTTCATTTGCATCTACCTTCACATCGGACGAGGGTTGTACTACGGCTCTTTCTTATATAA
R. catesbeiana      ··C·CC·A··A··············C·····A········T··T··C··T··T··TT·C·········C·········CC·C·············C·A·C·T·····
R. zhengi           ··C·CC·C·····C··T··········C··C··A·····················TT·C·····T······G··AC·C··T··T·····A·A·C·C·····
R. temporaria       ··C·CC····T·····T·············C··T··A·····T·····T··C··T······T·C···········G··CC·T··T········A·A·C·C··C··
R. dalmatina        ··C·CC····T··C··T·············C··C··A·····T·····T·····T··T··TT·C·················CC·T··T··········ATC·C·····
R. sauteri (Sanyi)  ·GC·CC·C·····C·····C·····C··G··A················C·········T·C··T·····G·······CC·T··T········C·A·C·C··C··
R. sauteri (Wulai)  ··C·CC·C·····C·············C··C··A························T·T·C··T········G··CC·T·············C·A·C·C·····
R. amurensis        ·GC·CC·A········T··C·····C··C··A········T·····C··T··T···T····T··T··G·····TC·T··T··T·····C·A·C·C·····
R. tagoi            ··C·CC·C·····C··T··C·····C··C··A··············C·········T·C·······T·········TC·T··T·············A·C·T·····
R. okinavana        ·GC·CC·C·····C··T··C·····C··C··A··············C·········T····T·····T·······TC·T··T·········C·A·C·C·····
R. tsushimensis     ··C·CC·C·····C·····C·····C··C··A························T·T·C··T········G··CC·T·············C·A·C·C·····
R. japonica         ··C·CC····T········C·····C··C·········T·······C··········TT·C·····T··G········CC·T··T········A·A·C·C··C··
R. ornativentris    ··C·TC·······C·····C·····C··C··A···········T··C··T··T··TT·C·················CC·T··T··T··T··C·A·C·C·····
R. pirica           ··C·TC·C·····C·····C·····C··C··A··············C··········TT·C··········G··G··CC·T··T··T·········A·C·T·····

                    201                                            250                                               300
X. laevis           AGAAACATGAAATATTGGTGTGATTCTTCTATTTTTAGTTATAGCTACAGCATTTGTAGGATATGTTCTACCATGAGGACAAATATCTTTTTGAGGGGCT
R. catesbeiana      ···············C·····A·····A··G········A···········T·········T··C···············C··············A···C·····C··C
R. zhengi           ·········G········A·····C··C·····CC····G···········C·····C·····C··C··C··C·········C··············A···C·····T··C
R. temporaria       ···G········C··C··A··A··C··CT····C·····G·····C······T·····C··C··C·····T··G·····G·······G··A·········T··C
R. dalmatina        ············C·····A··A··C··CT·G··CC····G··G··C······C·····G··C········T········G···············A···C·····T··C
R. sauteri (Sanyi)  ······T·····C··C··A··T·····C··C···C····G·····C······C··C·····C·····AT·G··G·············G··C···C·····C··C
R. sauteri (Wulai)  ·········G··C··C··G··T··C··C···C·C·····A··G··C······C·····G··C··C··C··G·····G··C················A···C·····C··C
R. amurensis        ···G········C········T··C···T····A·····A·····C······C··C·····C··C··A··G········C················A······G··C··C
R. tagoi            ············C··C··A·······C·····T········A··G··C······T··C·····C··C·····T········C··········G··A···C·····C··C
R. okinavana        ············C··C··A··C··C··C··C··CC····A·····C··G··T··C··G··T··C··C··G··G·····C··········G··A·········T···
R. tsushimensis     ······G··G··C··C··G··T··C··C···C·C·····A··G··C······C·····G··C··C··C··G·····G··C················A···C·····C··C
R. japonica         ···G········C··C··A··A··C··C··G··C·····A·····C······T·········C·····CT··········C··········G··C···C·····C···
R. ornativentris    ···G········C·····A··A··C··C······C····A·····C······T·········C·····GT··········C·················C···C·····C··C
R. pirica           ······G··G··C·····A··A··C··C··G··C·····G········C··T·········C······T····G·····C···G·····C···C·····C··C

                    301                                            350                                               400
X. laevis           ACAGTAATTACTAATCTTCTTTCTGCTATTCCGTACATCGGAAACGTACTAGTCCAATGAATTTGAGGAGGTTTCTCTGTAGATAACGCCACTTTAACCC
R. catesbeiana      ··············C·····C··A··CGCC··A········TTC··AC··T··T··············G·····T··A·····C·········CC·T····
R. zhengi           ··············C·····C··A···GCC··T···G······CT·AC·····T··············G··C·····A·····C·········CC·C··T·
R. temporaria       ··············C·····C··A··CGCC··C·····T··CT···AC··C········G··C··················A·····A·····C··T··T···C·T····
R. dalmatina        ·················C··C··A··CGCC··A········CC·A·AC··C··T········C·········C·····A·····C··T··A···C·C····
R. sauteri (Sanyi)  ···········C··C··C··C··A··CGCC··C·····T··CTC·AAC··C········G··C·····G··C·····A·····C·····A···C·T····
R. sauteri (Wulai)  ·····C··C·····C·····C··A··CGCC··C········CC···AC··T········G··C·····G··C·····G··G··C·····A··CC·G····
R. amurensis        ········C··C··C··C··C··A··CGCC··C········CTCT·AC··C···········C··G··G·········A·····················CC·C··T·
R. tagoi            ···········A·····C··C··A··CGCC··T·····T··CTC·AAC···········G··C·····G··C·····G·····C·····A···C·C····
R. okinavana        ·····T··C··C··C··C·····G··AGCC··C···G····TC···AC··C···········C·····C··C·····G·····C·····T··CC·C··T·
R. tsushimensis     ·····C··C·····C·····C··A··CGCC··C········CC···AC··T········G··C·····G··C·····G··G··C·····A··CC·G····
R. japonica         ···········C··C··C··C··A··CGCC··C··T·····CTC··AC··C··T········C·········C··T··A·····C·····A··CC·C····
R. ornativentris    ·····C·····A·····C··C··A··CGCC··C········CTTT·ACT····T······G·C··G·········C·····A·········T··T··CC·C····
R. pirica           ·················C··C··A··CGCC··C········CTTT·ACT·G···········C··G·········C·····G·····C·····T··CC·C····

                    401                                            450                                               500
X. laevis           GATTCTTCGCATTTCACTTCCTCCTTCCTTTTATTATTGCCGGAGCTAGCATTCTCCATCTTTTATTTCTCCACGAAACTGGATCAACAAACCCAACTGG
R. catesbeiana      ·······TA·T·········A····C··G········C··A·C···A··T··GA····C··GC·T··C·····TC·······C···T·C·········A··
R. zhengi           ········A····C······A····C··A···········T·CCATA··T··AA·T······C·····C··T··TC····A··G··CT·········T·A··
R. temporaria       ········A····C·····TA·T·····G·········C··T·CT·TG·····AA·T······C·········A···C····A··G···T·T·····C··C··
R. dalmatina        ·······TA·G··C······A····C··A·····C··C··T·CTATG·····AA·T··C···C·G··C··A··TC····A······T·C··T··T··C··
R. sauteri (Sanyi)  ·G······A···········A·T··C··A·····C··C··T·CT··C···G··A·T··C··CC·G·····T··TC·G··A······T·C·····C··A··
R. sauteri (Wulai)  ····T··TA·G··C······A····C··C·····C··C··T·CTATA·····GA····C··CC····C··G··TC····G·····CT·C·····C··A··
R. amurensis        ·G·····TA····C······A····C··A·····C··C···CC·T·····C·AA·T······C·····T··A··TC····A······T·T·····C··G··
R. tagoi            ········A····C······A·······G·····C··C··G·CT·TA·······A·T·····CC·G···T··A···C····A··C···T·T·····C··A··
R. okinavana        ·G··T··TA····C······A·T··C··C··C··C·····T·CG··A·····GA····C···C····C··G··TC····G··G···T·C·····C··A··
R. tsushimensis     ····T··TA·G··C······A····C··C···G·C··C··T·CTATA·····GA····C··CC····C··G··TC····G·····CT·C·····C··A··
R. japonica         ·······TA·T··C······A····C··A········C····CTATA·····AA·T··C··CC····C··G··TC····G·····CT·C·········A··
R. ornativentris    ·······TA····C·····TA·T··C··G·····C··C··A·CT·TA··T··AA····C··CC·······T··TC····C··C···T·C·········A··
R. pirica           ····T··TA····C······A····C··G··C··C··C··A·CTATA··T··AA·T··C··C··G·····T···C····A··C···T·C··T··C··A··

                    501                                            550
X. laevis           ATTAAACTCAGACCCAGATAAAGTACCTTTCCACCCATACTTCTCTTACAAAGACCTTTTAGGCTTCCTTATTATACTTACAGCACT
R. catesbeiana      CC·C··T···A··TT······G··CT·C··T··T···········C··········T·A··C······AC··········GG···CT·
R. zhengi           CC·C··T··CA··TTC········CA·C··T·····T········C············C··T······G·C···C····AGGCCTG··
R. temporaria       ·C·C·····TA·T·T···C·····CT·A····················T········C··C······G·····T·····GGT·····
R. dalmatina        ·C·C·····TA···T···C·····CT·A········G··T·····C···············C······G····CT·····GGT·····
R. sauteri (Sanyi)  GC·······TA··TT···C·····CT·A········C···········T········C··C······G·G··CC····GGG···T·C
R. sauteri (Wulai)  TC·CGG···CA···T·······A·CT·A·····T···········C···········C··T·····TG·GG·CT····CGGC··C··
R. amurensis        ·C·C·····TA···T···C·····TT·C··T·····C···················T··C··T·····TG·····T·····GGG··G··
R. tagoi            ·C·CG····TA···T·········CT·A········T········C···········C··T··T··TG·A··CC····CGG···T··
R. okinavana        ·C·T·····TA···T···C·····C··A········C········C···········C··T··T··TG·GGCCC·G··CGGG·····
R. tsushimensis     TC·C·G···CA···TG······A·CT·A·····T···········C···········C··T·····TG·GG·CT·····GGT··C··
R. japonica         ·C·T·····TA···T···C·····CT·A········C········C·········T··C··C·····TG·A··CC····GGGTA·C··
R. ornativentris    ·C·T·····CA·T·T···C·····CT·A········C········C·········T··C··C······G·A··CC·T···GGT··C··
R. pirica           ·C·C·····CA···T···C·····CT·G········T··T··T··C·T·······T··C··C·····TG·A··CC·T···GGT··C··
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made to confirm this idea. In order to assess the taxonomic position of *R. zhengi* and further ascertain the invalidity of *Pseudorana*, we tried in the present study to clarify the phylogenetic relationships of *R. zhengi* with ten species of brown frogs from Japan, Taiwan, Russia, and Europe on the basis of sequence variation in the mitochondrial cytochrome b gene.

MATERIALS AND METHODS

We extracted DNA from small amounts (less than 50 mg) of ethanol preserved tissue samples from liver and muscles of one *R. zhengi* from Sichuan, China. Tissues from specimens of *R. temporaria* and *R. dalmatina* from Czech and Austria were also used for the analyses (see appendix). We amplified a part of the mtDNA cytochrome b gene using the polymerase chain reaction (PCR) and sequenced approximately 600 bp of this gene following Tanaka et al. (1994, 1996). Primers used for the amplification and sequencing were: L14850-1 (5'-TCTCCGCATGATGAAACTTCGGCTC-3': Tanaka et al., 1994), L14850-2 (5'-TCTCATCCTGATGAAACTTTGGCTC-3': Tanaka et al., 1996), and H15502 (5'-GGATTAGCTGGTGTGAAATTGTCTGGG-3': Tanaka-Ueno et al., 1998b). We adopted the sequence numbering system of the human sequence (Anderson et al., 1981).

We estimated genetic distance among haplotypes based on the pairwise matrix of nucleotide sequence divergences calculated by Kimura's (1980) two-parameter method with the DNADIST program in PHYLIP Version 3.5c (Felsenstein, 1993). A distance tree was generated with the neighbor-joining algorithm (Saitou and Nei, 1987) in the NEIGHBOR program of the PHYLIP package (Felsenstein, 1993). For maximum likelihood (ML) analysis, we employed Felsenstein's (1993) DNAML algorithm with empirical base frequencies and an expected transition-transversion bias of 2.0. Degrees of support for internal branches in each tree were assessed by 1000 bootstrap pseudoreplications (Felsenstein, 1985). Maximum parsimony (MP) analysis of unweighted, unordered character-state data was performed with the heuristic search option using the tree bisection-reconnection (TBR) branch-swapping in PAUP* version 4.0b (Swofford, 1998). Confidence in each node was assessed by 1000 bootstrap replications.

For comparisons, we used the published sequences of two populations of *R. sauteri* (sensu Chou and Lin, 1997) from Taiwan, six species from Japan (*R. pirica*, *R. ornativentris*, *R. japonica*, *R. okinavana*, *R. tagoi*, and *R. tsushimensis*), one species from Russia (*R. amurensis*), and an outgroup taxon, *R. catesbeiana* (Tanaka-Ueno et al., 1998a, b). We also incorporated a published sequence of *Xenopus laevis* (Dunon-Bluteau et al., 1985) into the analysis as that for another outgroup.

RESULTS

We could constantly obtain nucleotide sequence data of 587 bp for all samples (Fig. 1). Samples of *R. dalmatina* from Czech and Austria showed an identical sequence. In the neighbor-joining tree (Fig. 2A), monophyly of *R. zhengi* and the brown frogs was supported in high iterations of 88.1%. Nevertheless, *R. zhengi* exhibited the earliest divergence among the ingroup taxa and the monophyly of the brown frogs was also supported in high iterations of 84.0%.

Among the brown frogs, two European species formed a cluster and diverged next

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FIG. 1. Aligned sequences of a 587 bp segment of the cytochrome b gene. Dots indicate identity with the sequence of *Xenopus laevis*.

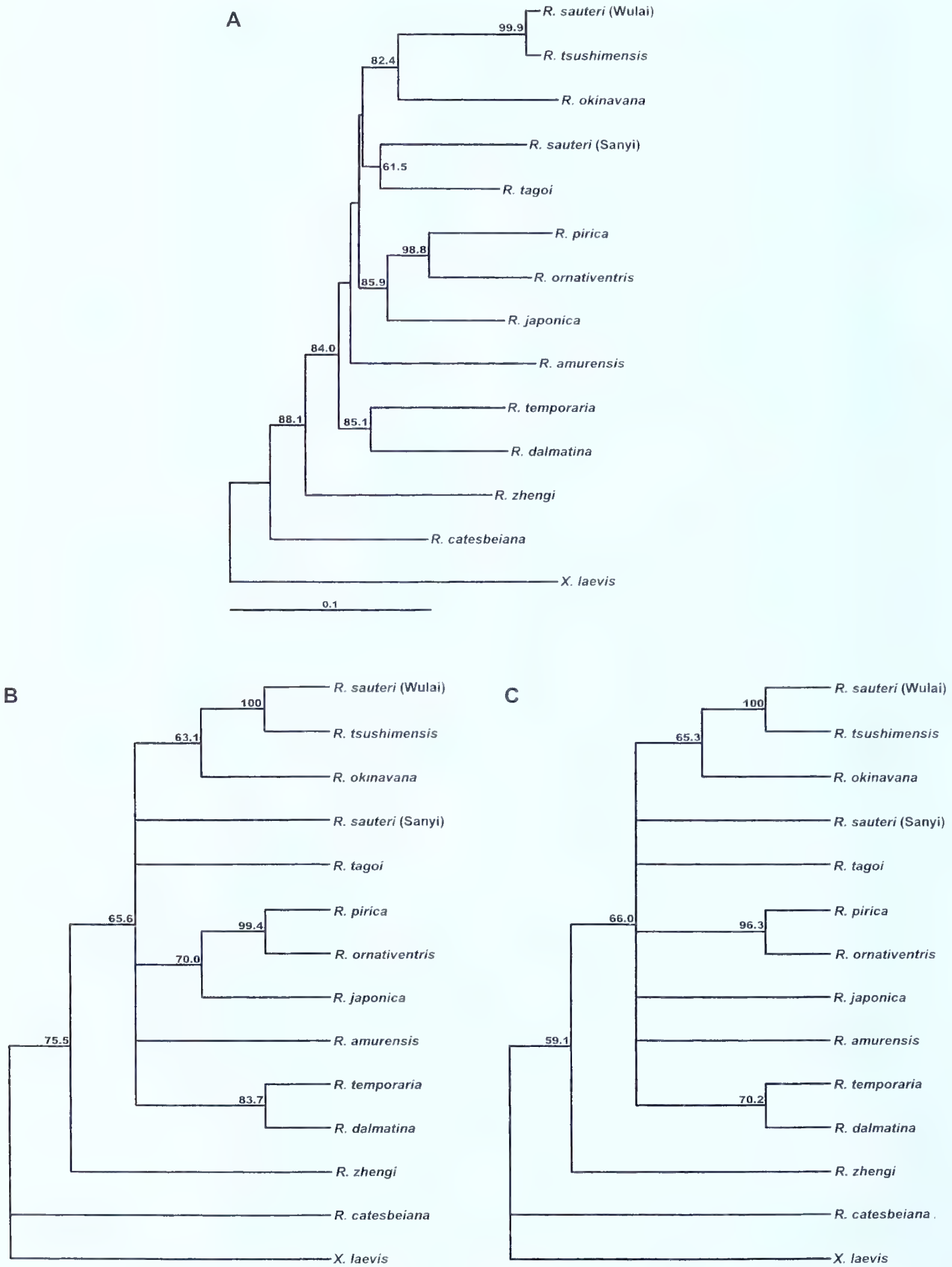

FIG. 2. A neighbor-joining tree rooted at the midpoint of the longest path (A), a maximum-likelihood tree (B), and a bootstrapped parsimony tree (C). Nodal values in all trees indicate percent support for branches in 1,000 bootstrap replicates (BP). BP values less than 50% are omitted.

to *R. zhengi*. The order of divergence among the remaining Asian brown frogs was the same as that reported by Tanaka-Ueno et al. (1998b). However, most of these branches were supported in very low iterations of less than 50%, and the relationships within the brown frogs were viewed as polytomous including five clusters. First, two European species formed a cluster (Group 1) supported in iterations of 85.1%. Then, *R. amurenis* diverged from all the remaining brown frogs (Group 2).

The latter consisted of two major clusters, of which one, supported by iterations of 85.9%, contained *R. japonica*, *R. pirica*, and *R. ornativentris* (Group 3). Within this group, sister relationships of *R. pirica* and *R. ornativentris*, both characterized by 2n=24 chromosomes against 2n=26 in most other brown frogs including *R. japonica* (Tanaka et al., 1996), was supported in high iterations of 98.8%.

The other major cluster contained two clusters, one consisting of *R. tagoi* and *R. sauteri* from Sanyi (Group 4: supported in 61.5% iterations), and the other of *R. okinavana*, *R. tsushimensis*, and *R. sauteri* from Wulai (Group 5: supported in 82.4% iterations). In Group 5, a sister relationship of *R. tsushimensis* and *R. sauteri* from Wulai was supported in very high iterations of 99.9%.

In this way, *R. zhengi* was found to be greatly diverged from the brown frogs including European species, and was not close to *R. sauteri* from Sanyi or Wulai.

In the maximum-likelihood tree (Fig. 2B), bootstrap supports of monophylies of *R. zhengi* and the brown frogs (75.5%), and of the brown frogs (65.6%) were weaker than in the neighbor-joining tree. Brown frogs exhibited polytomous relationships and the monophyly of *R. tagoi* and *R. sauteri* from Sanyi (Group 4 of the neighbor-joining tree) were supported in low iterations of 47.7%. Supports of monophylies of Groups 3 (70.0%) and 5 (63.1%) recognized in the neighbor-joining tree were also weaker, while those of the other groups were nearly the same as in the neighbor-joining tree.

In the maximum-parsimony tree (Fig. 2C), monophylies of *R. zhengi* and the brown frogs, and of the brown frogs were supported as in the neighbor-joining tree, although iterations were lower (59.1% and 66.0%, respectively). This analysis resulted in a polytomous consensus tree encompassing seven clades, and monophylies were supported only for Group 1 (supported by 70.2% iterations), Group 5 (65.3%), and the *R. pirica–R. ornativentris* clade of Group 3 (96.3%). The sister relationship of *R. tsushimensis* and *R. sauteri* from Wulai was supported by 100% iterations, whereas that of *R. japonica* with the species with 24 chromosomes (*R. pirica* and *R. ornativentris*) was not supported, unlike in the neighbor-joining tree.

DISCUSSION

From the results of the present study, monophylies of *R. zhengi* and the brown frogs, and of the brown frogs were supported. Nevertheless, relationships among the latter were not sufficiently resolved, and only sister relationships of (1) two European species (*R. temporaria* and *R. dalmatina*), (2) *R. tsushimensis* and *R. sauteri* from Wulai, (3) *R. okinavana* and the (2) species, and (4) *R. pirica* and *R. ornativentris* were unequivocally confirmed.

Also, it is highly probable that (5) *R. japonica* and the species with 24 chromosomes (*R. pirica* and *R. ornativentris*) are phylogenetically very close, and that *R. tagoi* is not remote from *R. sauteri* from Sanyi. On the other hand, relationships of *R. amurensis* with other brown frogs remain unclear, although the order of divergence among the Asian brown frogs in the neighbor-joining tree was identical with our previous results (Tanaka-Ueno et al., 1998b).

In all the three trees obtained, *R. zhengi* was located outside of the brown examined, and this clearly indicates an early divergence of the ancestral form of *R. zhengi* from the common ancestor of the brown frogs from both Europe and Asia, including *R. sauteri* from Taiwan (Chou and Lin, 1997; Tanaka-Ueno et al., 1998b).

We have already rejected Fei et al.'s (1990) and Dubois' (1992) idea of regarding *R. sauteri* from Taiwan as a member of a distinct genus or subgenus *Pseudorana*, and suggested that the species be included in the subgenus *Rana* of the genus *Rana* (Dubois, 1992) in our previous studies of the Taiwanese, Japanese, and Russian brown frogs (Tanaka-Ueno et al., 1998a, b). The present study again demonstrated a monophyly of the brown frogs including *R. sauteri*, and parallel divergences of this species in the course of brown frog evolution.

Rana sauteri is surely divergent from other brown frogs in having unique larval characteristics of complicate dentition on a large mouth and abdominal sucker in adaptation to a torrential life (Chou and Lin, 1997), but these characteristics can be viewed as results of convergent evolution (Tanaka-Ueno et al., 1998a).

Zhao (1999) first gave only a brief diagnosis of *R. zhengi* without providing pictures, and later redescribed it in longer form and showing a color photograph of two specimens that are not clearly designated as type series (Zhao, 2000a). In the latter paper, Zhao (2000a) also compared this species with *R. sangzhiensis* and *R. johnsi* under an implicit presumption of their close affinities to *R. zhengi*. These latter two species have been assigned to members of *Pseudorana* (Fei et al., 1990; Dubois, 1992).

Zhao (2000a) also described tadpoles of *R. zhengi* with rather complicate dentition (mostly I:4–4/4–4:I), but never mentioned the presence or absence of an abdominal sucker. *Rana johnsi*, which is now regarded as a distinct species (e.g., Inger et al., 1999), was first described as a subspecies of *R. sauteri* (see Smith, 1921) and is superficially similar to the latter species in adult morphology. However, larvae of this species lack an abdominal sucker or large mouth parts, although dentition is complicate (I:5–5/4–4:I; Matsui's observation of the type series in the Natural History Museum, London). Most probably, this species belongs to a lineage distant from brown frogs of the *R. temporaria* group.

In the same journal (Sichuan Journal of Zoology) as he described *R. zhengi* (Zhao, 1999, 2000a), Zhao (2000b) referred to convergent properties of *R. sauteri* with torrent-dwelling *Amolops* species in the structure of larval ventral sucker without mentioning *R. zhengi*. He should have described this type of modification in *R. zhengi* if it was ever present in this species.

Thus, both morphologically and genetically, *R. zhengi* is considered to be remote from *R. sauteri*. In order to clarify the exact phylogenetic position of *R. zhengi* in the genus *Rana*, it is necessary to examine biochemically representatives of many subgenera proposed by Dubois (1992), as well as morphologically related species such as *R. johnsi* and *R. sangzhiensis*.

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APPENDIX

Material examined.—Sources of tissue samples from a total of four frogs are stored at the Graduate School of Human and Environmental Studies, Kyoto University (KUHE).

Rana zhengi Zhao, 1999 (N=1): Zhangcun Village, Hongya Co., Sichuan Province, China (KUHE 27951).

Rana temporaria Linne, 1758 (N=1): Frahi Lazhe, Czech (KUHE 28259).

Rana dalmatina Bonaparte, 1840 (N=2): U Soukupu, the Czech Republic (KUHE 28260); Vienna, Austria (KUHE 26568).

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Advertisement Call Structures of Frogs from Southwestern India, with Some Ecological and Taxonomic Notes

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Abstract: Advertisement call structures of ten frog species, six ranids and four rhacophorids, from southwestern India were analyzed. *Rana temporalis, Limnonectes limnocharis, Indirana semipalmata, Nyctibatrachus major*, and *Philautus* cf. *bombayensis* had simple calls consisting either of a single note or of a series of similar notes. *Limnonectes* cf. *keralensis* and *P.* cf. *travancoricus* had a relatively long call with a well-differentiated first note, and *L. syhadrensis, P. leucorhinus*, and *Rhacophorus malabaricus* had complex call structures with a variety of call types. Several ecological notes associated with breeding, and some taxonomic problems are presented.

Key words: Advetisement call; bioacoustics; frogs; India; Western Ghats

INTRODUCTION

The acoustic characteristics of frog calls are one of the most important diagnostic features of species, and many species have been characterized acoustically or even described as new by the call structures (Kuramoto, 1985; Schneider and Sinsch, 1992). Calls play a critical role in reproductive ecology of frogs which rely on vocal communication rather than visual or olfactory cues (Wells, 1977; Roy, 1997). Recently it has been recognized that calls provide the most convenient and reliable evidence for the presence of certain species in surveys of anuran amphibians (Bridges and Dorcas, 2000).

The call structures of more than 20 Indian frogs have been analyzed (reviewed by Kanamadi, 1996; Roy, 1996). India has a rich amphibian fauna (Inger and Dutta, 1986) and more than 100 frog species occur in the Western Ghats (Daniels, 1992). There are many species which are very difficult to distinguish from each other and it is expected that acoustic features will provide useful means for identification.

In the present study we report advertisement call structures of ten frog species from southwestern India.

MATERIALS AND METHODS

Calls were recorded in the field during June and July 1999 and June 2000 in the following localities, all in Karnataka state: Kadri (Mangalore), Padil (Mangalore), Madikeri, Kudremukh, Bhatkal, and Talagini (about 35 km NE of Bhatkal). Madikeri, Kudremukh, and Talagini are in the Western Ghats.

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Recordings were made using a cassette tape recorder (Sony TCM-55 or TCM-AP5) and the calls were analyzed using the software SoundEdit Pro (Macromedia) for a Macintosh computer.

Of ten species analyzed, six belong to the Ranidae and four to the Rhacophoridae. For the Ranidae we adopted currently recognized genera (Dubois, 1992; Duellman, 1993). Identification of species is very difficult especially in the genera *Limnonectes* and *Philautus*. Some frogs did not fit the original descriptions of species which are known to occur in the Western Ghats and adjacent areas, and we refer to them as *Limnonectes* cf. *keralensis*, *Philautus* cf. *travancoricus*, and *P.* cf. *bombayensis* in the present study.

RESULTS AND DISCUSSION

1. Rana temporalis

Males of *R. temporalis* were calling on the banks of ditches or ponds, sometimes perching on branches. The advertisement call was composed of a single note of about 0.05 sec in duration and about 2.8 kHz in dominant frequency (Fig. 1A, Table 1). In several calls, fine harmonic structures were observed between 1 and 3 kHz. Mean interval between notes was about 1.7 sec. Within a series of single-note calls, there were calls with a few successive notes (Fig. 1B). We observed two males actively calling in close proximity on the bank of a pond in Madikeri. The notes were much shorter and repeated at a much higher rate than the ordinary calls, but we were unable to analyze this encounter or the agonistic calls due to heavy background noise.

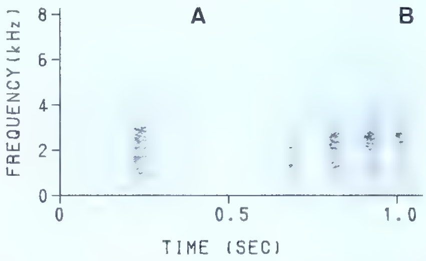

FIG. 1. Advertisement calls of *Rana temporalis* recorded in Kudremukh at 22°C. Single-note call (A) and multi-note call (B).

Note length and dominant frequency differed significantly between populations of Kudremukh and Madikeri (Table 1). This may be due to the differences in breeding urge and body size of males, but we could not get any concrete evidence.

2. Limnonectes limnocharis

The advertisement calls of this species were recorded in rice paddies of Padil on 6 July 1999 at an air temperature 26°C. The call was composed of many notes with a mean note length of 0.19 sec (N=11), a mean fundamental frequency of 2.07 kHz, and a second dominant band at 3.73 kHz (Fig. 2A). The duration of a call and the number of notes involved varied considerably from call to call. Two neighboring males tended to call alternately (Fig. 2A).

The distribution range of *L. limnocharis* is very wide, covering most parts of the

TABLE 1. Acoustic characteristics of the advertisement calls of *R. temporalis* (mean±SD).

Locality	N	Note length (sec)	Dom. frequency (kHz)	Temp.	Date
Kudremukh	15	0.046±0.008	2.73±0.10	22°C	3 July 1999
Madikeri	10	0.059±0.007	2.83±0.07	22°C	10 July 1999

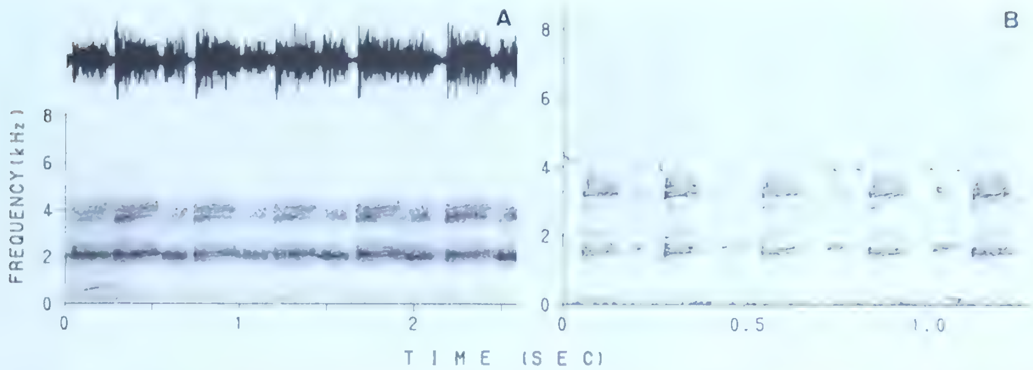

FIG. 2. A part of the advertisement call of *Limnonectes limnocharis* recorded in Padil at 26°C (A) and in Fukuoka, Japan at 21°C (B). Scales in B differ from those in the other figures because of different analyzing instruments.

Oriental region and its northeastern extremity reaches Japan (Inger, 1999; Zhao, 1999). The senior author has recorded advertisement calls of this species from Japan, the Ryukyu islands and Taiwan (Kuramoto, unpubl.). The general structure of calls from Indian and Japanese populations of *L. limnocharis* is similar, although there are remarkable differences in note repetition rate (Fig. 2B). The advertisement call structure of *L. limnocharis* from Thailand (Heyer, 1971) and China (Mou and Zhao, 1992) is similar to that obtained by us, but detailed comparative analysis of this species must await future studies.

The advertisement calls of *L. limnocharis* from Dharwad, Karnataka, reported by Kanamadi et al. (1995) are essentially identical with those recorded by us. Because call parameters, especially temporal features, vary with temperature, male body size, breeding urge, and interaction between calling males, direct comparisons of published data are often misleading. However, the mating calls of *L. limnocharis* from northeastern India (Assam and Meghalaya) (Roy and Elepfandt, 1993; Roy, 1996) and Nepal (Dubois, 1975) differ considerably from our results, especially in spectral features (dominant band at 1–2 kHz, in contrast to 2–4 kHz in the other populations). As the detailed taxonomic status of the *Limnonectes limnocharis* complex is still unsettled, there may be several cryptic species.

Males were calling in hollows or crevices in soil near the water's edge and it was difficult to find calling males. Males of Japanese *L. limnocharis* call on the ground or in shallow water while completely exposing themselves. Eggs were small (1.1–1.2 mm in diameter) and pigmented in both Indian and Japanese populations.

3. Limnonectes *cf.* keralensis

This frog (Fig. 3A) apparently belongs to the *Limnonectes limnocharis* complex and is very similar to *L. keralensis*, although it lacks the yellowish spot on the back which is characteristic of *L. keralensis* (Daniels, 1998). We found this frog in Kudremukh and Talagini. All specimens had a narrow mid-dorsal stripe and, from the posterior end of the mid-dorsal stripe, a thin line ran along the rear surface of the thigh to the foot. Males were 46–47 mm in SVL and a female was 58 mm.

The advertisement calls recorded in Kudremukh at 22°C (Fig. 4) consisted of 5–9 notes (mean 6.7, N=21), with a total call length of 0.91±0.12 sec (mean±SD). The mean note interval was 0.12±0.02

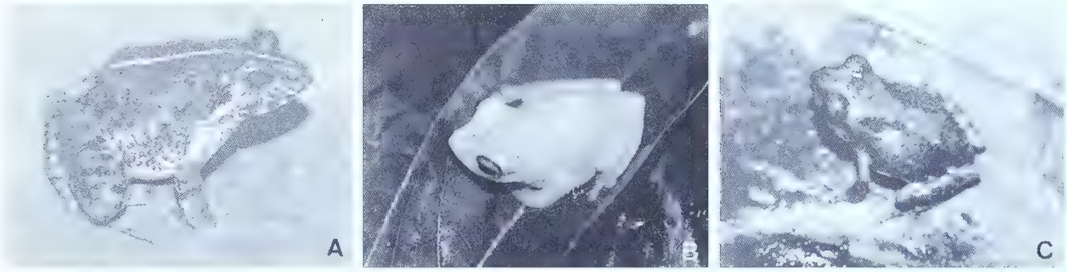

FIG. 3. *Limnonectes* cf. *keralensis* female from Talagini (A), *Philautus* cf. *travancoricus* male from Kudremukh (B), and *P.* cf. *bombayensis* male from Kudremukh (C).

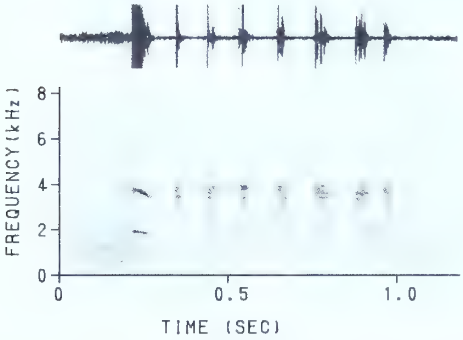

FIG. 4. An advertisement call of *Limnonectes* cf. *keralensis* recorded in Kudremukh at 22°C.

The first note (0.08 sec in duration) was longer than the rest and showed a clear harmonic spectral structure with a mean fundamental frequency of 1.73 kHz. The second harmonic band was dominant and this band corresponded to the dominant band of the rest of the notes (3.59±0.13 kHz). In some calls, frequency modulation in the first note (shown in Fig. 4) was not so obvious.

Males were calling on the banks of ditches. When two males were calling side by side they emitted advertisement calls alternately as in *L. limnocharis*. A female collected on 3 July 1999 had mature ova in the ovary; the ova were pigmented and small like those of *L. limnocharis*.

4. Limnonectes syhadrensis

The advertisement calls of this small species were recorded in Kadri, Padil, and Bhatkal. The calls from Kadri and Bhatkal consisted of a series of notes with a dominant frequency at about 3.8 kHz (Fig. 5A). The notes at the posterior portion of a call tended to be divided into several pulse groups. The number of notes and the note repetition rate were significantly greater in Bhatkal than in Kadri (Table 2). This may reflect a difference in breeding urge of the calling males.

The structure of calls recorded in Padil differed remarkably (Fig. 5B). The call consisted of a series of long notes (about 0.3 sec in duration) with fine spectral bands. Although we could not record the calls showing the transition from the multi-note call to the long-note call, it is probable that the finely pulsed notes at the posterior part of the former become fused or elongated to produce the latter.

Males were calling on the ground or among pebbles near the water edge. Apparently this frog lays eggs in temporary water bodies produced by rain, but the small water bodies thus produced may persist long enough for larval development during the rainy season.

A female collected in Padil (17.5 mm in SVL) laid 44 eggs in a bottle. The eggs were pigmented and about 1.4 mm in diameter. The jelly capsules were relatively hard and the capsules adhered to each other. The relatively large ovum size and the nature of the jelly capsules

TABLE 2. Acoustic characteristics of the multi-note type advertisement calls of *L. syhadrensis* (mean±SD).

Locality	N	Call duration (sec)	No. notes	Dom. frequency (kHz)	Temp.	Date
Kadri	8	1.06±0.21	11.4±2.9	3.79±0.04	27°C	7 June 2000
Bhatkal	6	1.21±0.14	17.8±4.0	3.83±0.07	27°C	26 June 2000

differed remarkably from those of *L. limnocharis* and *L.* cf. *keralensis*. The larvae were bottom dwellers and very stationary. When they moved they swam very quickly.

Dubois (1975) reported calls of *L. syhadrensis* from Nepal which resembled the calls of *L. limnocharis* and differed significantly from the present results. In the Nepal population, note length varied from 0.06 to 0.12 sec, but was nearly constant within a call. Compared with the body sizes of the type specimens from Bombay (SVL 27 mm in male and 31.5 mm in female; Annandale, 1919) and specimens from Nepal (28.5 mm in male and 33.7 mm in female; Dubois, 1975), all our specimens were much smaller (less than 20 mm. N=17 including both sexes). These remarkable differences strongly suggest the need of taxonomic reexamination of "*L. syhadrensis*" of south India.

5. *Indirana semipalmata*

The calls of this frogs were recorded in Madikeri on 30 June 1999 at 22°C.

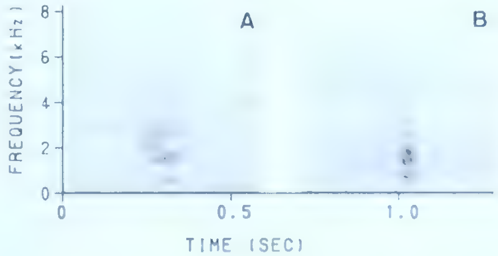

FIG. 6. An advertisement call of *Indirana semipalmata* recorded in Madikeri at 22°C (A) and an advertisement call of *Nyctibatrachus major* recorded in Talagini at 23°C (B).

This frog emitted calls consisting of a single note with a mean length of 0.13 sec and a dominant frequency band at about 1.59 kHz (N=13. Fig. 6A). Conspicuous frequency modulation was observed.

Kadadevaru et al. (2000) described the acoustic characteristics of *I. beddomii*. The call duration of *I. beddomii* (0.12 sec) is nearly the same as in *I. semipalmata*, but the call of *I. beddomii*

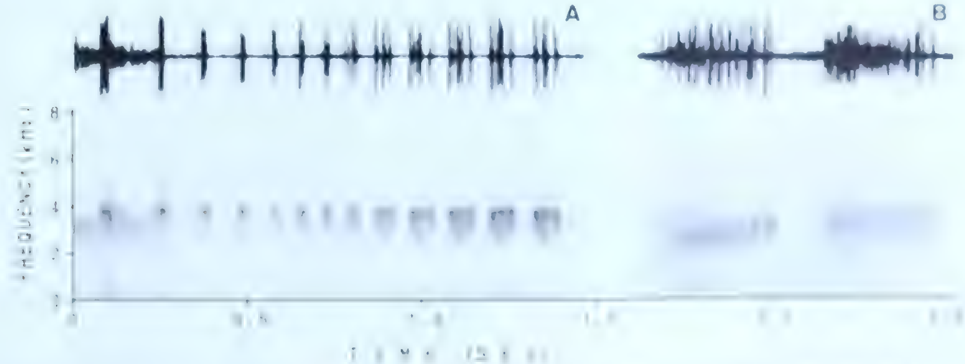

FIG. 5. Multi-note advertisement call (A) and long-note [illegible] call (B) of [illegible] *syhadrensis* recorded in Kadri at 27°C and in Padil at [illegible]°C, re[illegible]

consists of 10–14 pulses and lacks frequency modulation.

A male (28 mm in SVL) was calling in a depression on a cliff where a small quantity of water was seeping out. Many eggs were found around the depression. Undoubtedly the eggs were laid by several females. Embryos were in blastula stages (on 30 June 1999) and covered with tough, transparent jelly capsules. We carried a part of eggs to the laboratory and the embryos hatched three days after collection. The larvae had a long tail, tail length being about three times body length. A long tail is characteristic of the tadpoles of its congener *I. beddomii* (Boulenger, 1920).

6. Nyctibatrachus major

The advertisement calls of this species were recorded in a small stream in Talagini on 26 June 2000 at 23°C. The calls (Fig. 6B) consisted of a single note which was emitted at an interval of about 20 sec. Note length was 0.05 sec (N=7) and had a wide main frequency band ranging from 0.6 to 1.9 kHz. Conspicuous frequency modulation was observed in the upper part of this main spectral band. A more faint band was observed at about 2.43 kHz.

This species was calling in the shallow waters of streams. A female collected in Madikeri on 19 June 2000 (42.2 mm in SVL) laid six eggs in a bottle. The eggs were pigmented, 2.7 mm in ovum diameter and 4.1 mm in outer diameter of the egg capsules. Since this female had ova of various sizes in the ovary, from mature ova to smaller unpigmented ones, it seemed highly probable that this species lays eggs not in a single large clutch but in several small clutches at intervals of several days or several weeks. Males had very small testes relative to body size (mean testis length was 6.3% of SVL and was the smallest among 15 Indian frogs we examined), and this may be relevant to this reproductive mode.

7. Philautus leucorhinus

The advertisement calls were recorded in Kadri on 21 June 1999 (28°C) and 7 June 2000 (27°C), and in Padil on 28 June 1999 (temperature not recorded) and 10 June 2000 (24°C). We found this species also in Madikeri. This small arboreal frog emitted diverse calls among low vegetation in the forest and in house gardens.

The most common, and probably basic, advertisement call was composed of a short note which involved 3–9 (mean=5.6, N=20) pulse groups (Fig. 7A). The mean note length was 0.12 sec and the mean dominant frequency 3.23 kHz (28°C). From this call may derive a longer note (Fig.

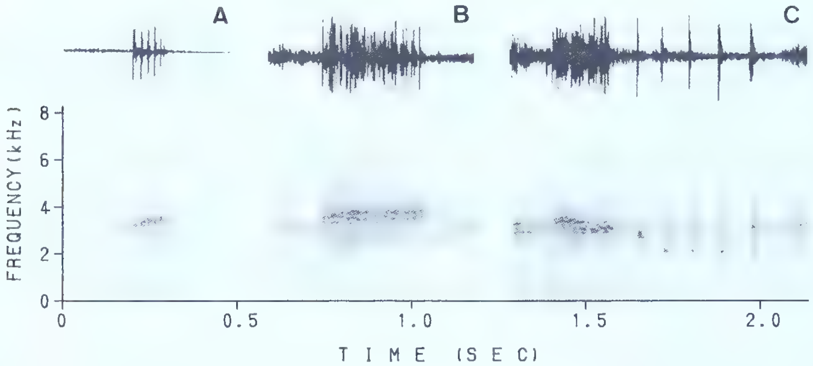

FIG. 7. Advertisement calls of *Philautus leucorhinus* recorded in Kadri on 21 June 1999 at 28°C (A) and on 7 June 2000 at 27°C (B and C).

7B), and its posterior portion is segregated to form distinct pulse groups with regular intervals (Fig. 7C). There were several calls composed only of these segregated pulse groups. In the segregated pulse groups the dominant frequency was lower (about 2.1 kHz) than in the basic note. The two call types of *P. leucorhinus* reported by Patil et al. (1996) apparently correspond to the first and last call types in the present study. Territorial and encounter calls of *P. leucorhinus* reported by Arak (1983) correspond to the segregated pulse groups in the present study.

Whether the different call types are emitted in different situations, or whether the different call types play different ecological roles is unknown. Arak (1983) analyzed vocal interaction between males in *P. leucorhinus* from Sri Lanka.

In general, calls of Indian rhacophorid frogs have complex structures involving basically two phases, fast and slow. Although advertisement call structures of several rhacophorid species of east Asia are available for comparison (Heyer, 1971; Matsui et al.; 1986; Lue et al., 1995), only *Rhacophorus owstoni, R. moltrechti*, and *R. prasinatus* were reported to have complex calls with fast and slow phases (Kuramoto, 1975, 1986; Chou, 1992).

8. Philautus *cf.* travancoricus

This species (Fig. 3B) fits the original description of *Philautus travancoricus* (as *Ixalus travancoricus*) by Boulenger (1891) except that our specimens lacked "larger black dots scattered on the back and on the tibia". Three male specimens we collected were 24.1, 25.0 and 27.4 mm in SVL. Calls were recorded in Madikeri on 29 June 1999 (22–23°C) and 10 July 1999 (21°C), and in Kudremukh on 3 July 1999 (21°C).

The advertisement calls were relatively long with an initial short note composed of several pulses and many slowly repeating pulses (Fig. 8). The two parts are designated here as fast phase and slow phase, respectively. The call duration was variable because the number of pulses in the slow phase varied extensively (Table 3). The dominant frequency did not differ between the two phases and there were fine harmonic structures around the dominant band (Fig. 8). The males were calling from branches or leaves in the forest; the perching site seemed somewhat higher than in *P. leucorhinus*, because we found most males of *P.* cf. *travancoricus* above

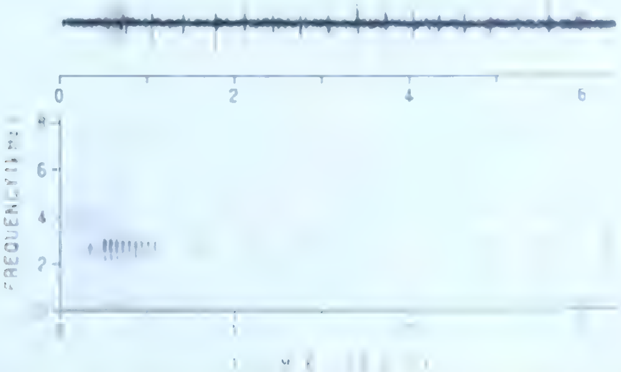

FIG. 8. Advertisement calls of *Philautus* cf. *travancoricus* recorded in Kudremukh at [illegible] (spectral pattern) and in Madikeri at 23°C (wave form). Time [illegible] the whole call structure.

TABLE 3. Acoustic characteristics of the advertisement calls of *Philautus* cf. *travancoricus* (mean±SD, with range in parentheses).

Locality		Call duration	Fast phase		Slow phase		Frequency
(Temp.)	N	(sec)	Duration (sec)	N of pulses	Duration (sec)	N of pulses	(kHz)
Madikeri	12	12.3±17.5	0.34±0.05	8.3±1.1	11.7±17.4	44.8±75.4	2.70±0.08
(22–23°C)		(3.0–65.0)	(0.28–0.45)	(6–10)	(2.3–64.2)	(4–275)	(2.57–2.83)
Madikeri	17	9.5±14.8	0.36±0.04	10.5±1.1	8.9±14.9	31.2±59.2	2.74±0.05
(21°C)		(1.9–62.4)	(0.28–0.44)	(9–13)	(1.3–62.3)	(4–249)	(2.61–2.86)
Kudremukh	22	4.1±1.5	0.37±0.06	10.4±1.8	3.5±1.55	10.9±4.4	2.68±0.12
(21°C)		(1.3–8.1)	(0.30–0.50)	(8–16)	(0.8–7.5)	(3–23)	(2.45–2.87)

our height, whereas males of *P. leucorhinus* were easily found below our eyes. This type of call with a specialized first note is rather common in the genera *Afrixalus* and *Hyperolius* of the African tree frogs (Hyperoliidae) (Schiøtz, 1999).

9. Philautus *cf.* bombayensis

This very small frog with a mean SVL of 18.1 mm (17.4–18.8 mm, N=6) in males is dark brown in dorsal color (Fig. 3C). The tympanum is inconspicuous, webbing between toes is not developed, and large pale markings (yellow in life) are evident on the anterior side of the thigh. The combination of these characteristics fits the original description of *P. bombayensis* (Annandale, 1919; as *Ixalus bombayensis*). However, *P. bombayensis* was reported to be 30 mm in SVL and to have a papilla on the tongue (Annandale, 1919; Ahl, 1931). We found that the male frogs (females were not available) have a large process in the middle portion of the humerus, extending antero-ventrally. This bony process is easily recognizable by stroking the underside of the upper arm with forceps.

Recordings were made in Kudremukh on 3 July 1999 (22°C) and 14 June 2000 (24°C). Males were calling from among low vegetation, often less than 50 cm above ground. The advertisement call was a series of short notes, 0.034 sec in duration and 3.95 kHz in dominant frequency (Fig. 9). No harmonic structures were observed. The note was repeated rather irregularly, at about 1–2 sec intervals. Occasionally two notes were emitted successively at about a 0.26 sec interval (Fig. 9).

This call was very sharp and loud. Daniels (1998) described the call of *P. variabilis* as "a loud metallic 'tink; tink-tink'". Although this description of the call agrees completely with the call of the present species, the smaller body size and rudimentary webbing clearly discriminate this species from *P. variabilis*. Günther (1858) examined osteological characters in the original description of *P. variabilis* (as *Ixalus variabilis*), but did not mention the peculiar process on humerus, so it is evident that *P. variabilis* lacks this process.

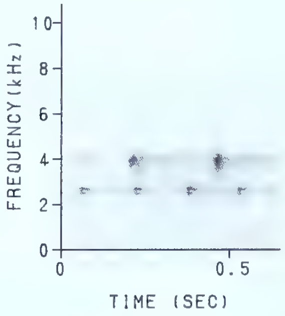

FIG. 9. An advertisement call of *Philautus* cf. *bombayensis* recorded in Kudremukh at 24°C. This call is composed of two successive notes. The sound with dominant band slightly less than 3 kHz is that of an insect.

In the genus *Philautus*, the call structure of only three species, *P. parvulus* from Thailand, *P. annandalii* from Nepal and *P. leucorhinus* from Sri Lanka, has been reported (Heyer, 1971; Dubois, 1976; Arak, 1983). The call of *P. parvulus* is simple, consisting of a series of several short pulses. Probably, the call structures of *P.* cf. *bombayensis* and *P. parvulus* are primitive in the genus, from which more complex call structures may be derived.

10. Rhacophorus malabaricus

The advertisement calls of *R. malabaricus* were recorded in Madikeri on 29 June 1999 at an air temperature of 23°C. The basic call was composed of several pulses repeated relatively slowly and with low dominant frequencies (Fig. 10). The call duration was 0.91±0.37 sec (N=15) and the dominant band was at 1.69±0.14 kHz. The number of pulses varied from five to 14, 7.9 on average, and were repeated at mean intervals of 0.14 sec. Very often this call was accompanied by a short note with a fast pulse repetition rate (mean interval 0.05 sec) and less energy (Fig. 10). This accompanying note was also observed in the Japanese green tree frog *R. arboreus* (Kuramoto, 1975).

In the complex advertisement calls of this species, a few pulse groups followed the basic call (Fig. 11). The basic part (slow phase) was longer than the basic call (1.65±0.23 sec, N=8) due to the increase of pulses (13.6±1.8), and pulse intervals became longer toward the end of the phase (Fig. 11). The number of accompanying fast pulse groups (fast phase) varied from one to four in our recordings. The mean duration of the fast notes was 0.36 sec, the mean number of pulses involved in a note was 16.0, and pulses were repeated at a mean interval of 0.024 sec, about 6 times faster than the slow phase. Obviously the spectral composition of the fast phase differed significantly from that of the slow phase; the energy was concentrated between 1 and 3 kHz without showing clear dominant bands. The note in the fast phase is apparently related to the accompa-

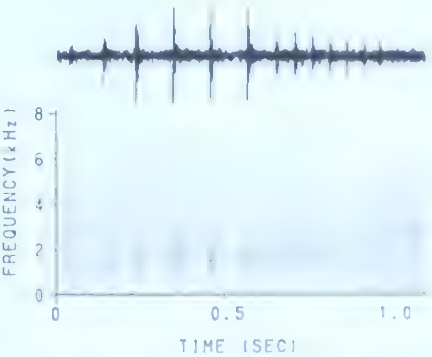

FIG. 10. An advertisement call of *Rhacophorus malabaricus* recorded in Madikeri at 23°C. This call consists of five pulses and accompanies a note with fast-repeating seven pulses

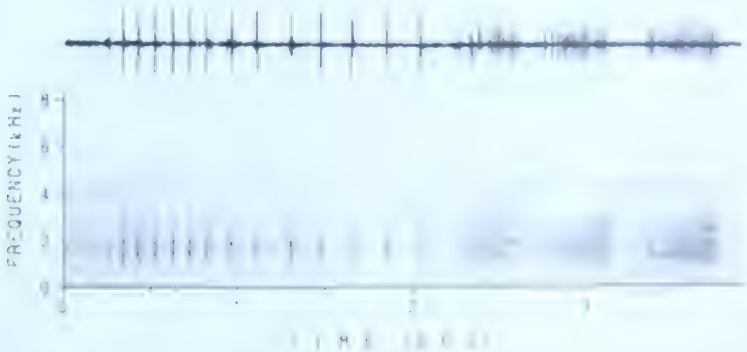

FIG. 11. A complex advertisement call of *Rhacophorus malabaricus* recorded in Madikeri at 23°C.

nying note of the basic call, but in the latter pulse repetition rate was much slower and the spectral composition was similar to the pulses of the main part of the basic call. The complex calls were emitted where several males occur in a relatively high density. Probably vocal interactions may be intensified in such a situation.

Males were calling on the edge of a small artificial pond, and we observed many foam nests at the water edge. We observed many tadpoles in the pond and found metamorphosed froglets, too. Apparently breeding of this frog begins in early June or May in this area, and lasts relatively long.

The complex call of *R. malabaricus* resembles the advertisement calls of *R. owstoni* and *R. moltrechti* reported by Kuramoto (1975, 1986), but the basic call is lacking in the latter two species.

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Diet Composition of the Indian Rice Frog, *Rana limnocharis*, in Rice Fields of Central Japan

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Abstract: *Rana limnocharis* from rice fields of Nara, central Japan, fed on a wide variety of prey animals. Arthropods constituted 94.8% in number and 52.9% in volume of total prey items. Among prey taxa, ants (26.9%) comprised the largest proportion in number, and earthworms (36.2%) in volume. One froglet and one tadpole were also recovered from frog stomachs. The generalized food habits of *R. limnocharis* would ensure the establishment of populations of this species outside its natural range.

Key words: *Rana limnocharis*; Food habits; Insectivore; Artificial introduction; Colonization

INTRODUCTION

Rana limnocharis is one of the most broadly distributed species of Asian anurans, ranging from East Asia through Southeast Asia to India (Maeda and Matsui, 1999). In Japan, this species is distributed in the central Ryukyus, Kyushu, Shikoku, and an area from the Chugoku District to the Tokai District of Honshu (Maeda and Matsui, 1999). Other than one exotic species, *R. catesbeiana*, *R. limnocharis* is the only amphibian species distributed in both the Ryukyu Archipelago and the mainislands of Japan.

Rana limnocharis is considered to have been artificially introduced into various parts of the Kanto District during the last decade (Osawa, 1998; Hasegawa and Ogano, 1998; Hayashi et al., 2000; see Hayashi and Kimura [2001] for review). The introduced *R. limnocharis* may have an unfavorable influence on indigenous anuran populations of each locality. However, *R. limnocharis* from Japan has never been studied from a viewpoint of community ecology: currently available information relevant to the natural history of this frog only concerns its embryonic temperature tolerances (Kuramoto, 1967), growth (Shichi et al., 1988), and vocalizations (Hata and Nagoshi, 1995). The paucity of studies concerning ecological relationships of coexisting anuran species makes it difficult for us to predict the effects of introduced *R. limnocharis* on the native anuran populations.

Food is an important niche axis for partitioning coexisting anurans (Toft, 1985), and food [illegible] overlap (or diet similarity) has been used to hypothesize potential interactions between coexisting species (Inger and Greenberg, 1966; Stewart and Sandison, 1972; Miller, 1978; Licht, 1986; McAlpine and Dilworth, 1989; Werner et al., 1995). In the mainislands of Japan, food habits have been studied for *R. nigromaculata*, *R. porosa brevipoda*, *R. rugosa*, and *Hyla japonica* inhabiting rice fields (Hirai and Matsui, 1999; 2000a, b, 2001a, b). With respect to food habits of Japanese populations of *R. limnocharis*, however, a list of prey items recovered from four individuals (Tomida, 1976) is the only available information to the present.

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In this paper, we present diet composition of *R. limnocharis* inhabiting rice fields of central Japan, and compare it with published data for conspecific populations from outside of Japan.

METHODS

We collected frogs from rice paddy fields in Nara City, Nara Prefecture, central Japan (34°42'N, 135°51'E). Collections were conducted at night between 2100 h and 2300 h on 20 June, 14 July, and 6 August 1997. Immediately after we captured frogs, we extracted their stomach contents by forced regurgitation with forceps, and preserved the contents in 10% buffered formalin for later identification and analysis. For each frog, we measured snout–vent length (SVL), mouth width and body mass, and examined the presence or absence of male secondary sexual characters (nuptial pads, vocal sacs, and an M-shaped dark marking on the throat). Individuals were divided into three sex-age classes: juvenile, adult male, and adult female. Adult males are easily differentiated from the others in possessing the male secondary sexual characters, but adult females and juveniles, both lacking those characters, are not distinguishable from each other with certainty unless the gonads are inspected by dissection. Nevertheless, because females are known to reach sexual maturity at larger body size than males (Maeda and Matsui, 1999), for we practical purposes, we regarded individuals smaller than the smallest adult male (actually 32.2 mm in SVL) as juveniles, and larger individuals as adult females, including sub-adults. In addition, we clipped toes in unique combinations for individual identification. After these procedures, we released frogs where they were captured.

In the laboratory, we identified stomach contents to the lowest practical taxonomic level, and measured maximum length (L) and width (W) of each prey item to the nearest 0.1 mm using either a caliper or a calibrated ocular micrometer fitted to a dissecting microscope. For partially digested prey items, we estimated lengths by measuring width and then using predetermined length-width regressions from intact prey (see Hirai and Matsui [2001c] for more details). Volume (V) of each prey item was calculated using the formula for an ellipsoid:

$$V=4/3\pi(L/2)(W/2)^2$$

RESULTS

Diet composition

Forty frogs (17 adult females, 15 adult males, eight juveniles) were captured, of which six (five adult females, one adult male) had empty stomachs. The bulk of the diet was represented by three invertebrate phyla (Arthropoda, Mollusca, and Annelida), with Arthropoda including four classes (Insecta, Araneae, Crustacea, and Diplopoda), making up 94.8% in number and 52.9% in volume of total prey items (Table 1). Insecta contained seven orders, and comprised 62.3% in number and 31.0% in volume. Ants (Formicidae) comprised the largest proportion in number (26.9%), followed by spiders (Araneae; 20.3%) and dipterans (18.4%). In volume, however, earthworms (Oligochaeta; 36.2%) predominated in the

TABLE 1. Diet composition of *Rana limnocharis*, sampled from juveniles (J: 80 prey from eight frogs, total volume 332.18 mm^3), adult males (M: 69 prey from 14 frogs, total volume 1874.95 mm^3), and adult females (F: 63 prey from 12 frogs, total volume 1991.85 mm^3).

	Frequency of occurrence (%)				Numeric proportion (%)				Volumetric proportion (%)			
Prey taxa	J	M	F	Total	J	M	F	Total	J	M	F	Total
Arthropoda												
Insecta												
Hymenoptera												
Formicidae	87.5	35.7	33.3	47.1	50.0	14.5	11.1	26.9	7.9	0.7	1.0	1.4
Non-Formicidae	12.5	0.0	0.0	2.9	1.3	0.0	0.0	0.5	0.8	0.0	0.0	0.1
Coleoptera	12.5	0.0	33.3	14.7	1.3	0.0	7.9	2.8	0.1	0.0	21.0	10.0
Larvae	12.5	7.1	0.0	5.9	1.3	1.5	0.0	0.9	0.3	0.5	0.0	0.2
Diptera	50.0	35.7	50.0	38.2	5.0	31.9	20.6	18.4	0.6	1.8	0.9	1.3
Larvae	12.5	7.1	8.3	8.8	1.3	1.5	3.2	1.9	0.3	0.2	0.2	0.2
Lepidoptera	0.0	14.3	0.0	5.9	0.0	2.9	0.0	0.9	0.0	1.8	0.0	0.8
Larvae	0.0	0.0	16.7	5.9	0.0	0.0	3.2	0.9	0.0	0.0	1.9	0.9
Hemiptera	25.0	28.6	8.3	20.6	2.5	7.3	1.6	3.8	1.5	7.7	1.1	4.1
Dermaptera	12.5	0.0	0.0	2.9	1.3	0.0	0.0	0.5	21.6	0.0	0.0	1.7
Orthoptera	12.5	21.4	25.0	20.6	2.5	4.4	7.9	4.7	14.2	1.8	17.7	10.3
Arachnida												
Araneae	50.0	57.1	66.7	58.8	13.8	23.2	25.4	20.3	10.8	9.8	3.0	6.6
Crustacea												
Isopoda	37.5	21.4	25.0	26.5	8.8	4.4	9.5	7.6	32.9	11.2	14.9	14.6
Amphipoda	0.0	7.1	0.0	2.9	0.0	1.5	0.0	0.5	0.0	0.7	0.0	0.3
Diplopoda	25.0	7.1	8.3	11.8	8.8	1.5	1.6	4.3	3.1	0.1	0.2	0.4
Mollusca												
Gastropoda	12.5	14.3	16.7	17.7	2.5	2.9	3.2	2.8	6.0	0.2	3.1	2.0
Annelida												
Oligochaeta	0.0	7.1	16.7	8.8	0.0	1.5	3.2	1.4	0.0	52.6	25.7	36.2
Vertebrate												
Amphibia												
Anura	0.0	0.0	8.3	2.9	0.0	0.0	1.6	0.5	0.0	0.0	8.5	4.1
Larvae	0.0	7.1	0.0	2.9	0.0	1.5	0.0	0.5	0.0	11.0	0.0	4.9
Plant materials	37.5	50.0	33.3	41.2	—	—	—	—	—	—	—	—
Minerals	12.5	16.7	0.0	8.8	—	—	—	—	—	—	—	—

diet, followed by woodlice (Isopoda; 14.6%) and orthopterans (10.3%), and ants comprised a small proportion (1.4%).

Besides invertebrates, a recently metamorphosed froglet of *Hyla japonica* and an unidentified tadpole were recovered from the stomachs of medium-sized individuals (41.2 mm, and 37.2 mm in SVL, respectively). Plant materials and minerals also occurred in 41.2%, and 8.8%, respectively, of the stomachs examined. When food items were classified by their habitats, aquatic organisms (dipteran larvae, gerrid water-striders, amphipods, gastropods exclusive of land-snails and slugs, and anuran larvae) occurred in 32.4% of frog stomachs, but comprised only 6.1% and 9.3% in number and volume, respectively.

Comparisons among sex-age classes

Adult females, significantly larger than adult males in all measurements (SVL, mouth width, and body mass: Mann-Whitney U-test, $p<0.01$ for all, see Table 2), tended to take larger prey on average than did adult males and juveniles. Even so, significant difference was detected only between adult females and juveniles (Dunn's multiple comparison test, $p<0.05$). Although diet composition varied among sex-age classes (Table 1), only the frequency of ants showed statistically significant difference in the between-class comparisons: it was significantly higher in juveniles (87.5%) than in adult males (35.7%) and adult females (33.3%) (χ^2-contingency table test, df=2, $\chi^2=6.88$, $p<0.05$). Absence of significant differences in the number and volume of prey items among the three classes (Dunn's multiple comparison test, $p>0.05$), may be attributable to the within-class large variation, and/or small sample size for each class.

DISCUSSION

This study revealed that *R. limnocharis* from central Japan feeds predominantly on arthropods like most other ranid frogs hitherto studied (e.g., Jenssen and Klimstra, 1966; Houston, 1973; Whitaker et al., 1983; Hirai and Matsui, 1999). According to the past studies on the populations of *R. limnocharis* outside of Japan, arthropods comprised 89–100% in number of total prey items in the diet (Liu and Chen, 1933; Berry, 1965; Monhanthy-Hejmadi and Acharya, 1982). The value obtained by our study (94.8%) falls within this range.

Among diverse arthropod prey taxa, ants were reported to comprise the largest proportion in rice fields of China (27%; Liu and Chen, 1933), and in the swamps of Singapore (28%; Berry, 1965). These reported proportions are quite similar to the value in our result (26.9%). The previous studies, however, did not examine diet variations between juvenile and adult *R. limnocharis*. As shown in this study, the numeric proportion of ants is much larger in juveniles than in adults. This

TABLE 2. Means (±SD) of character dimensions of frogs and prey. Sample size and range are given in parentheses.

	Juveniles	Adult males	Adult females
Frogs			
SVL (mm)	29.3±2.7	35.8±2.9	41.4±3.5
	(8: 24.4–31.2)	(15: 32.2–40.2)	(17: 34.8–46.5)
Mouth width (mm)	10.1±0.9	11.9±1.0	13.6±1.3
	(8: 8.6–10.9)	(15: 10.9–14.0)	(17: 11.4–16.0)
Body mass (g)	1.9±0.4	3.6±0.9	6.2±1.6
	(8: 1.1–2.3)	(15: 2.4–5.6)	(17: 3.6–9.7)
Prey			
Length (mm)	3.6±0.9	5.0±1.5	7.6±4.9
	(7: 2.5–4.9)	(9: 3.3–8.3)	(9: 3.4–19.4)
Number/stomach	10.0±8.6	4.9±4.8	5.3±4.0
	(8: 2–30)	(14: 1–20)	(12: 1–13)
Volume/stomach (mm^3)	40.3±48.6	133.9±257.1	166.0±137.1
	(8: 0.7–149.0)	(14: 3.4–985.6)	(12: 5.4–384.7)

indicates that when diet composition is compared among populations, diet variation within a population should be considered.

Nevertheless, even if this kind of variation is considered, diet composition may differ among localities. For example, beetles (41%) were the most numerically predominant prey in India (Monhanthy-Hejmadi and Acharya, 1982) unlike in the other three localities. The second most important prey was hemipterans (16%) in China (Liu and Chen, 1933), termites (18%) in Singapore (Berry, 1965), ants (13%) in India (Monhanthy-Hejmadi and Acharya, 1982), and spiders (20.3%) in this study site. These local variations strongly suggest that the food habits of *R. limnocharis* are generalized, and that the frog changes diet menus flexibly in response to local variation in frequency of available prey items.

It is noteworthy that *R. limnocharis* consumed a tadpole and a froglet in this study site. Much larger frogs like the bullfrog (*R. catesbeiana*) often eat small vertebrates (see Bury and Whelan [1984] for review), but there are few reports that relatively small frogs like *R. limnocharis* feed on vertebrate prey. Even in *R. nigromaculata* and *R. porosa brevipoda*, only large individuals (SVL>53 mm) eat syntopic froglets (Hirai and Matsui, 1999; 2001a). Therefore, it may be safe to state that the food habits of *R. limnocharis* are characterized by diverse diet composition from tiny insects to small vertebrates. Such food habits seem to contribute at least partially to the success of this frog in establishing feral populations through artificial introduction (Osawa, 1998; Hasegawa and Ogano, 1998; Hayashi et al., 2000), because the generalized food habits of *R. limnocharis* should be advantageous in dealing with unfamiliar environments where diversity and abundance of prey may greatly differ from the original habitat.

The recently introduced populations of *R. limnocharis* in the Kanto District have received a great deal of attention (Osawa, 1998; Hasegawa and Ogano, 1998; Hayashi et al., 2000), because its colonization may lead to unfavorable influence on the native anuran populations. Further research is urgently needed to evaluate such influence and to determine whether or not to exterminate the introduced *R. limnocharis* for conservation of the native anurans. Since the colonized areas are still limited (Osawa, 1998; Hayashi and Kimura, 2001), extermination would be still possible.

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Phylogenetic Relationships of Geoemydine Turtles (Reptilia: Bataguridae)

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Abstract: Monophyly of the batagurid subfamily Geoemydinae was evaluated, and phylogenetic relationships within the subfamily were inferred on the basis of 35 morphological characters. Two approaches, parsimony analysis using the branch and bound algorithm, and neighbor-joining clustering of an absolute distance matrix, were used. The results of these analyses yielded phylograms that were almost identical in branching topology, and poorly supported the monophyly of Geoemydinae. This subfamily thus seems to be a metataxon, most likely consisting of the sister group of Batagurinae (*Geoemyda* group) and a more primitive stock of Bataguridae (*Mauremys* group). The latter accommodates *Mauremys* and *Sacalia* and its monophyly is not well supported. The former consists of the remaining ten geoemydine genera united by two synapomorphies—absence or reduction of the quadratojugal, and posteriorly short-sided anterior neurals. Relationships revealed by our analyses necessitate some changes in the generic classification of Geoemydinae. First of all, *Cistoclemmys* Gray, 1863 (type species: *Ci. flavomarginata*, often synonymized to *Cuora* Gray, 1855 (type species: *Cu. amboinensis*), is shown to be a valid genus closely related to *Pyxidea* and *Geoemyda* rather than to *Cuora* (sensu stricto). *Rhinoclemmys* Fitzinger, 1835 (type species: *R. punctularia*) is shown to be polyphyletic, and *Chelopus* Cope, 1870 (type species: *C. rubida*), is resurrected to accommodate *R. rubida* and *R. annulata*. The plastral hinge seems to have evolved three times in the Bataguridae—in the *Cyclemys-Notochelys* clade, *Cuora* (sensu stricto), and the *Cistoclemmys-Pyxidea* clade.

Key words: Geoemydinae; Bataguridae; Phylogeny; *Geoemyda* group; *Mauremys* group

INTRODUCTION

McDowell (1964) recognized three subfamilies, Batagurinae, Emydinae, and Testudininae, in the family Testudinidae. Gaffney and Meylan (1988) raised these

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subfamilies to separate families (Bataguridae, Emydidae, and Testudinidae, respectively) of the superfamily Testudinoidea, making the Bataguridae the largest and most divergent family in the order Testudines. They also divided this family into two subfamilies, Batagurinae and Geoemydinae (Gaffney and Meylan, 1988). The former currently contains 22 species of 12 genera (David, 1994; McCord and Iverson, 1994) distributed over most of temperate to tropical Asia, except for the western part (Iverson, 1992). The latter consists of 45 species of 12 genera (David, 1994; McCord et al., 1995, 2000; Fritz et al., 1997; Fritz and Wischuf, 1997; McCord, 1997), occurring in northern Africa, southern Europe, most parts of temperate to tropical Asia, central America, and northern South America (Iverson, 1992).

The first attempt to clarify the phylogeny of the Bataguridae was made by Hirayama (1984) (as the Batagurinae: see above), utilizing cladistic analyses of 37 species representing all recognized genera mainly on the basis of morphological characters. Two major groups were recognized, of which one, consisting of *Batagur*, *Callagur*, *Chinemys*, *Geoclemys*, *Hardella*, *Hieremys*, *Kachuga*, *Malayemys*, *Morenia*, *Ocadia*, *Orlitia*, and *Siebenrockiella*, was characterized by relatively broad triturating surfaces of jaws (=Batagurinae sensu Gaffney and Meylan, 1988), whereas the other, accommodating *Cistoclemmys*, *Cuora*, *Cyclemys*, *Geoemyda*, *Heosemys*, *Mauremys*, *Melanochelys*, *Notochelys*, *Pyxidea*, *Rhinoclemmys* and *Sacalia*, was characterized by relatively narrow triturating surfaces of jaws (=Geoemydinae). Hirayama (1984), while defining the broad triturating surfaces as a synapomorph of the Batagurinae, regarded the narrow triturating surfaces in the Geoemydinae as a primitive condition of the Bataguridae. He thus considered Geoemydinae as paraphyletic, with the *Rhinoclemmys annulata*–*R. rubida*–*Geoemyda*–*Cistoclemmys*–*Pyxidea* clade being monophyletic with the family Testudinidae rather than with the other geoemydine genera. In this hypothesis, the Batagurinae is the sister group of the Geoemydinae plus the Testudinidae, and the Bataguridae is also paraphyletic.

With respect to batagurine monophyly and phylogeny within this subfamily, Gaffney and Meylan (1988) approved Hirayama's (1984) view because most characters Hirayama employed show a low degree of homoplasy in their cladogram. They also shared the view of possible non-monophyly of the Bataguridae with Hirayama, but doubted the geoemydine phylogeny hypothesized by Hirayama because of a very high degree of homoplasy postulated therein. Since then, no comprehensive systematic studies have been carried out for the Bataguridae, leaving both the geoemydine phylogeny and the batagurid monophyly problematic.

Hirayama's (1984) paper was essentially an abstract of a much larger work of character analysis (not yet published), and thus a number of important issues, such as detailed descriptions of character states, were omitted (Gaffney and Meylan, 1988). In addition, a number of taxonomic changes have been made for geoemydine turtles since Hirayama's (1984) analysis (David, 1994; Obst and Reimann, 1994; McCord et al., 1995; Yasukawa et al., 1996; Fritz and Wischuf, 1997; Fritz et al., 1997; Iverson and McCord, 1997a; McCord, 1997; Lehr et al., 1998), though a few recent authors suspect some of these changes to have been misled by artificial hybrids and thus are untenable (van Dijk, 2000; Shi and Parham, 2001; Das, personal communication). These conditions have made it strongly desirable to conduct additional analyses for the geoemydine phylogeny with detailed methodological descriptions and considerations of those taxonomic changes. We therefore analyzed geoemydine phylogeny on the basis of the morphological characters of 32 species representing 11 genera (i.e., more than 70% of extant species representing all recognized genera but one).

MATERIALS AND METHODS

Representatives of the following genera were examined (see appendix for further details).

Cistoclemmys and *Cuora*.—At present, the genus *Cistoclemmys* Gray, 1863 (type species: *Ci. flavomarginata*), is usually regarded as a junior synonym of *Cuora* Gray, 1856 (type species: *Cu. amboinensis*) (Sites et al., 1984; Ernst and Barbour, 1989; McCord and Iverson, 1991; Iverson, 1992; David, 1994; Fritz and Obst, 1997; Wu et al., 1998). Nevertheless, several authors regard *Cistoclemmys*, also accommodating *Ci. galbinifrons*, as valid (e.g., Bour, 1980; Hirayama, 1984; Gaffney and Meylan, 1988; King and Burke, 1989; Ota, 1991; Zhao and Adler, 1993; Yasukawa and Ota, 1999). We tentatively followed the latter classification because of differences between the two species of *Cistoclemmys* and the remainder of *Cuora* sensu lato (*Cuora* sensu stricto) (Hirayama, 1984; Yasukawa, 1997), and examined the following species as their representatives—*Cistoclemmys flavomarginata* (13 specimens), *Ci. galbinifrons* (7), *Cuora amboinensis* (15), *Cu. aurocapitata* (4), *Cu. mccordi* (6), *Cu. pani* (4), *Cu. trifasciata* (5), and *Cu. zhoui* (4).

Among the five subspecies of *Ci. galbinifrons*, *Ci. g. serrata* is most divergent, exhibiting a weak pair of lateral keels and serration at the posterior margin of the carapace (Fritz and Obst, 1997), whereas the other four subspecies are difficult to distinguish from each other (Iverson and McCord, 1992a; Obst and Reimann, 1994; Lehr et al., 1998). Fritz and Obst (1997) suspected full-species status of *Ci. g. serrata*. Our specimens of *Ci. galbinifrons* lacked lateral keels or carapace serration (i.e., not belong to *Ci. g. serrata*), but were not identified to subspecies level.

There were no essential differences in characters examined among *Cuora trifasciata*, *Cu. aurocapitata* and *Cu. pani*, although these species differ remarkably from each other in coloration. They are probably very closely related to each other within *Cuora* (McCord and Iverson, 1991). We thus combined them into a single operational taxonomic unit (OTU) as the *Cuora trifasciata* species-group.

Cyclemys.—Fritz et al. (1997) described *Cyclemys pulchristriata* from Vietnam, and recognized four species, *C. dentata*, *C. oldhamii*, *C. pulchristriata*, and *C. tcheponensis*, for this genus mainly on the basis of differences in coloration. On the other hand, Iverson and McCord (1997a), analyzing morphometric variation within *Cyclemys*, described *C. atripons* from southeastern Thailand and Cambodia, and recognized only two species, *C. dentata* and *C. atripons*, for this genus. As such, the taxonomy of *Cyclemys* is still considerably confused, strongly requiring further verification. We examined 11 specimens of *Cyclemys*, but most of them were skeletal specimens and thus were not identified to the species level. Detecting no essential differences in all examined characters, we treated all of them as a single sample, *Cyclemys*, in this analysis.

Geoemyda.—This is a name-bearing type genus of the subfamily Geoemydinae. This genus was long regarded as consisting solely of *Geoemyda spengleri*, with two subspecies, *G. s. spengleri* and *G. s. japonica* (e.g., Ernst and Barbour, 1989; Iverson, 1992). Yasukawa et al. (1992), however, elevated both of them to full species, *G. spengleri* and *G. japonica*, because of their remarkable morphological divergences and prominent geographic isolation from each other. Moll et al., (1986), on the basis of comparisons with *G. spengleri* sensu lato, *Heosemys grandis*, and *H. spinosa*, reassigned *Heosemys silvatica* to *Geoemyda*. We followed this generic arrangement like a few other recent revisions (David, 1994; McCord et al., 1995; Yasukawa and Ota, 1999).

McCord et al. (1995) also assigned their new species, *Geoemyda yuwonoi*, and the two other species, *Heosemys depressa* and *H. leytensis*, to *Geoemyda*. However, generic status of the three species remains controversial (Fritz and Obst, 1996; Iverson and McCord, 1997b; Yasukawa and Ota, 1999). Recently, McCord et al. (2000) assigned *G. yuwonoi* to a new genus, *Leucocephalon*, and reassigned *G. depressa* to *Heosemys* on the basis of analysis of mitochondrial DNA variation. Since we did not examine those three species, we tentatively followed McCord et al. (2000). For the genus *Geoemyda* as defined above, *G. japonica* (13), *G. silvatica* (6), and *G. spengleri* (12) were examined.

Heosemys.—McDowell (1964) assigned five species, *Heosemys grandis*, *H. spinosa*, *H. depressa*, *G. leytensis* and *G. silvatica*, to *Heosemys*, probably on the basis of absence of the quadratojugal (squamosal in his terminology). He, however, did not directly examine the latter three species, of which *H. leytensis* and *H. silvatica* were recently reassigned to *Geoemyda* (see above). We examined *H. grandis* (3) and *H. spinosa* (4).

Leucocephalon.—This genus contains only one species, *Leucocephalon yuwonoi*, which was formerly assigned to *Geoemyda* or *Heosemys* (McCord et al., 2000). We have had no chance to examine this species.

Mauremys.—This genus currently consists of eight species, *M. annamensis*, *M. caspica*, *M. iversoni*, *M. japonica*, *M. leprosa*, *M. mutica*, *M. pritchardi*, and *M. rivulata* (Fritz and Wischuf, 1997; McCord, 1997), of which six, *M. annamensis* (6), *M. caspica* (1), *M. japonica* (10), *M. leprosa* (2), *M. mutica* (11), and *M. rivulata* (6), were examined. There were no essential differences in any character among *M. caspica*, *M. leprosa*, and *M. rivulata*. They were formerly considered as three subspecies of *M. caspica*, and are most likely monophyletic (Busack and Ernst, 1980; Ernst and Barbour, 1989; Iverson and McCord, 1994). We thus treat them as constituting a single OTU, the *Mauremys leprosa* species-group.

Melanochelys.—This genus contains two species, *M. tricarinata* and *M. trijuga*, of which only *M. trijuga* (10) was examined.

Notochelys.—We examined six specimens of *N. platynota*, the only representative of this monotypic genus.

Pyxidea.—This is a monotypic genus consisting solely of *P. mouhotii*. This species, formerly assigned to *Geoemyda* (e.g., McDowell, 1964) or *Cyclemys* (e.g., Wermuth and Mertens, 1961; Pritchard, 1979), is treated as *Pyxidea* by most recent authors (e.g., Hirayama, 1984; Ernst and Barbour, 1989; Iverson, 1992; David, 1994). Five specimens were examined.

Rhinoclemmys.—This is the only batagurid genus occurring in central and South America, and includes nine species: *annulata*, *areolata*, *diademata*, *funerea*, *melanosterna*, *nasuta*, *pulcherrima*, *punctularia*, and *rubida* (David 1994). Hirayama (1984) suggested the polyphyly of the genus, but all subsequent authors have apparently ignored this account (e.g., Ernst and Barbour, 1989; Iverson, 1992; David, 1994). Therefore, we tentatively treated them as composing a single genus, and examined *R. annulata* (2), *R. areolata* (2), *R. funerea* (3), *R. pulcherrima*, (5), *R. punctularia* (4), and *R. rubida* (1).

Sacalia.—Iverson and McCord (1992b) recognized three species, *S. bealei*, *S. quadriocellata*, and *S. pseudocellata*, for this genus. They showed some differences in coloration and shell shape, but none in internal morphology. We examined *S. bealei* (2), *S. quadriocellata* (2), and *Sacalia* sp. (2: skeletal specimens not identified at the species level). Because no essential differences existed among these specimens in any character, we treat them as a single OTU, *Sacalia*.

Batagurinae.—To evaluate states of characters in ingroup taxa and test their monophyly, we examined 44 batagurine specimens of 12 species representing all of the currently recognized batagurine genera: *Batagur baska* (2 specimens), *Callagur borneoensis* (3), *Chinemys reevesii* (10), *Geoclemys hamiltonii* (2), *Hardella thurjii* (3), *Hieremys annandalii* (3), *Kachuga smithii* (5), *Malayemys subtrijuga* (4), *Morenia petersi* (2), *Ocadia sinensis* (4), *Orlitia borneensis* (1), and *Siebenrockiella crassicollis* (5) (see "Specimens Examined" for further details). Das (2001) divided the genus *Kachuga* and assigned *K. smithii* and other three congeneric species to a separate genus, *Pangshura* Gray, 1869, usually regarded as a junior synonym or a subgenus of *Kachuga* (Wermuth and Mertens, 1977; Moll, 1987). However, we tentatively treated the two genera as a single unit, genus *Kachuga*, because both genera are possibly valid but probably monophyletic (Hirayama, 1984; Moll, 1987). In the "Characters Examined and Distribution of Their States" section, we refer to these species only by their generic name. We selected two batagurine genera, *Orlitia* and *Siebenrockiella*, as the possibly closest outgroup of the Geoemydinae, because these genera exhibited a particular similarity, as batagurines, with the latter by having narrow, ridgeless triturating surfaces, and lacking the secondary palate. *Orlitia* and *Siebenrockiella*, possibly closely related to each other, are likely to be regarded as the most primitive batagurines (McDowell, 1964; Carr and Bickham, 1986; Hirayama, 1984; Gaffney and Meylan, 1988).

We analyzed 35 characters: 20 for the skull, one for the hyoid apparatus, ten for the shell, one for the pelvic girdle, and three for soft parts). Several characters employed by McDowell (1964) and Hirayama (1984) were not used in our analyses, because they showed high intraspecific and/or intrageneric variation in ingroup taxa. These characters include: the shape of the fissura ethmoidalis, scutellation of the forearm, web of the hindlimb digits, and subdivision of the foramen nervi trigeminalis. See "Characters Examined and Distribution of Their States" for further details.

A species-character state matrix (Table 1) was analyzed using the Branch and Bound algorithm of PAUP (version 3.1.1., Swofford, 1991) to find all parsimonious networks, which were rooted by the outgroup species. All characters were set as ordered condition and were equally weighted. Three kinds of indices, consistency index (Kluge and Farris, 1969), retention index (Farris, 1989a), and rescaled consistency index (Farris, 1989b), were calculated for goodness-of-fit statistics.

In addition, we applied a neighbor-joining method (Saitou and Nei, 1987) for an absolute distance matrix using the NEIGHBOR program in PHYLIP (version 3.5c, Felsenstein, 1989) following Hikida (1993). The network calculated was also rooted by the outgroup species. This analysis was aimed at evaluating the validity of the relationships proposed in parsimonious trees and to assess the degrees of differentiation among samples. Since all characters were set as ordered condition in this study, the absolute distance (diff [y, z]) was calculated as diff (y, z)=y-z following Swofford (1991). The resultant absolute distance matrix is given in Table 2.

CHARACTERS EXAMINED AND DISTRIBUTION OF THEIR STATES

We examined states of 35 characters as below for our 26 geoemydine and 12 batagurine samples (Table 1)

Characters Used for the Phylogenetic Analysis

TABLE 1. Species-character state matrix for the subfamily Geoemydinae and its relatives.

	Characters																																		
										1										2										3					
Samples	1	2	3	4	5	6	7	8	9	0	1	2	3	4	5	6	7	8	9	0	1	2	3	4	5	6	7	8	9	0	1	2	3	4	5
Geoemydinae																																			
Cistoclemmys flavomarginata	0	2	1	1	1	1	?	?	2	0	0	1	1	1	0	0	2	2	0	1	2	2	0	0	1	1	1	2	2	1	1	0	0	1	0
Cistoclemmys galbinifrons	0	1	1	1	0	0	1	1	1	0	0	1	1	0	1	0	0	1	0	1	2	2	0	0	1	1	1	2	2	1	1	0	0	1	0
Cuora amboinensis	1	0	0	0	0	0	1	1	1	0	1	0	0	2	2	0	0	2	1	0	2	2	0	0	1	1	0	2	2	1	1	0	0	0	0
Cuora mccordi	0	0	0	0	0	0	1	1	2	0	0	0	0	2	2	1	0	2	1	0	1	1	0	0	1	0	0	2	2	1	1	0	0	0	0
Cuora trifasciata species group	0	0	0	0	0	0	1	1	2	0	0	0	0	2	2	1	0	2	1	0	1	0	0	0	1	0	0	2	2	1	1	0	0	0	0
Cuora zhoui	0	0	0	0	0	0	1	1	2	0	0	0	0	2	2	0	0	2	1	0	1	0	0	0	1	0	0	2	2	1	1	0	0	0	0
Cyclemys spp.	0	0	0	0	0	0	1	1	0	0	0	0	0	0	0	0	0	0	0	0	1	0	1	0	1	0	0	2	1	2	0	1	0	0	0
Geoemyda japonica	0	2	2	1	1	0	1	0	2	1	0	1	1	0	2	0	1	2	0	1	0	0	1	1	1	0	1	1	1	1	1	0	1	0	0
Geoemyda silvatica	0	1	1	1	1	1	?	?	2	1	0	1	1	0	1	0	2	0	0	1	0	0	0	1	1	0	1	1	1	1	0	0	1	0	0
Geoemyda spengleri	0	2	2	1	1	0	0	0	2	1	0	1	1	0	2	0	1	1	0	1	0	0	1	1	1	0	1	1	1	1	1	0	1	0	0
Hepsemys grandis	2	0	0	0	0	1	?	?	0	0	0	0	0	0	0	0	0	0	0	0	0	0	1	0	1	0	0	1	1	0	0	1	0	0	0
Heosemys spinosa	0	0	0	0	0	1	?	?	0	0	0	0	0	0	0	0	0	0	0	0	0	0	1	0	1	0	0	1	1	0	0	1	0	0	0
Mauremys annamensis	0	0	0	0	0	0	0	0	0	0	0	0	0	1	2	0	0	0	0	0	0	0	0	0	0	0	0	0	0	1	1	0	0	0	0
Mauremys japonica	0	0	0	0	0	0	0	0	1	0	0	0	0	1	1	0	0	0	0	0	0	0	1	0	0	0	0	1	1	1	1	0	0	0	0
Mauremys leprosa species group	0	0	0	0	0	0	0	0	0	0	0	0	0	1	1	0	0	0	0	0	0	0	0	0	0	0	0	1	1	1	1	0	0	0	0
Mauremys mutica	0	0	0	0	0	0	0	0	0	0	0	0	0	1	2	0	0	0	0	0	0	0	0	0	0	0	0	1	1	1	1	0	0	0	0
Notochelys platynota	0	0	0	0	0	0	1	1	0	0	0	0	0	0	0	0	0	0	0	0	1	0	1	0	0	0	0	2	1	2	0	0	0	0	0
Melanochelys trijuga	0	0	0	0	0	0	1	1	0	0	0	0	0	0	1	0	0	2	0	0	0	0	0	1	1	0	0	1	1	1	0	0	0	0	0
Pyxidea mouhotii	0	2	1	1	1	0	1	1	2	0	0	1	1	0	0	0	0	2	0	1	1	0	1	1	1	0	1	2	1	1	0	0	0	0	0
Rhinoclemmys annulata	0	0	0	0	0	0	1	1	2	0	1	0	1	1	1	0	0	2	0	1	0	0	0	0	1	0	0	1	1	1	1	0	0	0	0
Rhinoclemmys areolata	0	0	0	0	0	0	1	0	0	0	1	0	0	2	2	0	0	2	0	0	0	0	0	0	1	0	0	1	1	1	1	0	0	0	0
Rhinoclemmys funerea	1	0	0	0	0	0	1	0	0	0	1	0	0	2	2	0	0	2	0	0	0	0	0	0	1	0	0	1	1	2	1	0	0	0	1
Rhinoclemmys pulcherrima	1	0	0	0	0	0	1	0	0	0	1	0	0	2	2	0	0	2	0	0	0	0	0	0	1	0	0	1	1	1	1	0	0	0	0
Rhinoclemmys punctularia	2	0	0	0	0	0	1	0	0	0	1	0	0	2	2	0	0	2	0	0	0	0	0	0	1	0	0	1	1	2	1	0	0	0	1
Rhinoclemmys rubida	0	0	0	0	0	0	1	1	2	0	0	0	1	0	2	0	0	2	0	1	0	0	0	0	1	1	0	1	1	1	1	0	0	0	0
Sacalia spp.	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	1	1	1	1	0	0	0	0
Batagurinae																																			
Orlitia borneensi	2	0	0	0	0	0	0	0	0	0	0	1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	1	1	0	0	0	0	0
Siebenrockiella crassicollis	1	0	0	0	0	0	0	0	0	0	0	1	0	0	0	0	0	0	0	0	0	0	1	0	0	0	0	0	0	1	0	0	0	0	0

1. Frontal exposure onto orbital rim

In *Chinemys*, *Hieremys*, *Malayemys*, *Morenia*, and *Orlitia* of the batagurines, and *Heosemys grandis* and *Rhinoclemmys punctularia* of the geoemydines, the frontal was separated from the orbital rim by a strong connection between the prefrontal and postorbital. This condition was sometimes observed in *Siebenrockiella*, *Rhinoclemmys pulcherrima*, *R. funerea*, and *Cuora amboinensis*. In the other species, the frontal usually reached the orbital rim, but was sometimes separated by a very weak connection. We coded this character as: (0) usually present, without prefrontal-postorbital connection; (1) sometimes

TABLE 2. Absolute distance matrix for species of the subfamily Geoemydinae and its relatives.

	Characters																										
Samples	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21	22	23	24	25	26	27
1 *Cistoclemmys flavomarginata*																											
2 *Cistoclemmys galbinifrons*	9																										
3 *Cuora amboinensis*	20	15																									
4 *Cuora mccordi*	21	18	7																								
5 *Cuora trifasciata* species-group	22	19	8	1																							
6 *Cuora zhoui*	21	18	7	2	1																						
7 *Cyclemys* spp.	27	20	19	16	15	14																					
8 *Geoemyda japonica*	18	20	29	24	23	22	26																				
9 *Geoemyda silvatica*	18	17	30	25	24	23	21	8																			
10 *Geoemyda spengleri*	19	20	31	26	25	24	26	2	7																		
11 *Hepsemys grandis*	30	25	22	21	20	19	7	26	20	25																	
12 *Heosemys spinosa*	28	23	22	19	18	17	5	24	18	23	2																
13 *Mauremys annamensis*	29	24	19	16	15	14	14	24	21	22	13	11															
14 *Mauremys japonica*	26	21	18	15	14	13	11	21	18	19	10	8	5														
15 *Mauremys leprosa* species-group	26	21	18	15	14	13	11	23	18	21	10	8	3	2													
16 *Mauremys mutica*	27	22	17	14	13	12	12	22	19	20	11	9	2	3	1												
17 *Melanochelys trijuga*	26	19	16	13	12	11	9	19	16	21	10	8	11	10	8	9											
18 *Notochelys platynota*	27	20	19	16	15	14	2	26	21	26	9	7	12	9	9	10	9										
19 *Pyxidea mouhotii*	12	13	24	19	18	17	17	11	12	13	22	20	27	22	24	25	16	17									
20 *Rhinoclemmys annulata*	20	17	14	11	10	9	15	17	16	19	16	14	13	10	10	11	8	15	16								
21 *Rhinoclemmys areolata*	26	22	11	10	9	8	14	20	22	22	14	12	8	9	7	6	7	14	23	7							
22 *Rhinoclemmys funerea*	29	25	12	13	12	11	15	23	25	25	15	15	11	12	10	9	10	15	26	10	3						
23 *Rhinoclemmys pulcherrima*	27	23	10	11	10	9	15	21	23	23	13	13	9	10	8	7	8	15	24	8	1	2					
24 *Rhinoclemmys punctularia*	30	26	13	14	13	12	16	24	26	26	14	16	12	13	11	10	11	16	27	11	4	1	3				
25 *Rhinoclemmys rubida*	20	15	14	11	10	9	15	15	16	17	16	14	13	12	12	11	8	15	16	4	9	12	10	13			
26 *Sacalia* spp.	26	21	20	17	16	15	9	23	18	21	8	6	5	4	2	3	8	7	22	12	9	12	10	13	12		
27 *Orlitia borneensis*	29	24	23	22	21	20	12	26	19	24	7	9	8	9	7	8	11	10	23	17	14	15	13	14	17	5	
28 *Siebenrockiella crassicollis*	30	25	24	23	22	21	11	25	20	23	8	8	7	8	8	9	12	9	22	18	15	16	14	17	18	6	3

absent, with weak prefrontal-postorbital connection; (2) absent, with strong prefrontal-postorbital connection.

2. *Processus inferior parietalis*

The right and left processus inferior parietalis were almost parallel in most batagurids. In *Cistoclemmys flavomarginata*, *Geoemyda japonica*, *G. spengleri*, and *Pyxidea*, the processus were close to each other ventromedially, and the lower part of the cranial cavity was strongly convergent. *Cistoclemmys galbinifrons* and *Geoemyda silvatica* had the cranial cavity weakly convergent ventrally. We coded this character as: (0) almost parallel; (1) weakly approximating; (2) approximating.

3. *Foramen nervi vidiani*

The foramen nervi vidiani was located anteriorly to the anteroventral part of the processus inferior parietalis, and was bordered by the processus and palatine in most batagurid species. In *Geoclemmys*, *Hardella*, *Morenia*, *Cistoclemmys*, *Geoemyda*, and *Pyxidea*, the foramen was located posterolaterally or laterally to the anteroventral part of the processus inferior parietalis, bordered by the parietal, pterygoid, and epipterygoid, or sometimes open in the pterygoid (2/3 of *Geoemyda japonica* and 3/5 of *G. spengleri*). We coded this character as: (0) bordered by processus inferior parietal and palatine; (1) bordered by parietalis, pterygoid, and epipterygoid; (2) usually on pterygoid.

4. Medial process of jugal

In most batagurid species, the medial process of the jugal was moderately to well developed, and its ventromedial tip was expanded. In *Cistoclemmys*, *Geoemyda*, and *Pyxidea*, the process was weak and had a tapering ventromedial tip. We coded this character as: (0) moderate to well developed; (1) weak.

5. Contact between jugal and pterygoid

Most batagurid species had a medial process of the jugal which was firmly connected with the pterygoid. In *Cistoclemmys flavomarginata*, *Geoemyda*, and *Pyxidea*, the connection was usually absent or very weak if present. We coded this character as: (0) present; (1) usually absent or very weak.

6. Quadratojugal

Among batagurines, *Callagur*, *Batagur*, *Chinemys*, *Geoclemmys*, *Hardella*, *Malayemys*, and *Orlitia* had a massive quadratojugal, whereas in *Kachuga*, *Morenia*, *Ocadia*, and *Siebenrockiella* the quadratojugal was small to moderate in size. On the other hand, most geoemydines had a much smaller quadratojugal, although this element was moderate in size in *Mauremys* and *Sacalia*, and was exceptionally elongated but laterally strongly compressed in *Geoemyda spengleri*. *Hieremys*, *Cistoclemmys flavomarginata*, *Geoemyda silvatica*, and *Heosemys* had no quadratojugal at all. We coded this character as: (0) present; (1) absent.

7. Contact between quadratojugal and squamosal

In batagurines exclusive of *Hieremys*, the quadratojugal was firmly connected to the squamosal as well as to the quadrate and the jugal. On the other hand, the quadratojugal was absent (see above), or if present, did not usually contact the squamosal in most geoemydines. In *Geoemyda spengleri*, *Mauremys* and *Sacalia*, however, a weak contact was usually present. We coded this character as: (0) usually present; (1) absent; (?) no quadratojugal.

8. Contact between jugal and quadratojugal

The contact between the jugal and the quadratojugal was present in batagurines except for *Hieremys*, while it was usually absent in the geoemydines. A weak contact was present in *Geoemyda japonica*, *G. spengleri*, *Mauremys*, *Rhinoclemmys areolata*, *R. funerea*, *R. pulcherrima*, *R. punctularia*, and *Sacalia*. We coded this character as: (0) present; (1) absent; (?) no quadratojugal.

9. Bony beak of upper jaw

Most batagurid turtles had a shallow notch on the anteroventral margin of the unhooked bony beak composing the upper jaw. The corresponding portion was usually flat, or sometimes only weakly notched, with or without making the anterior portion hook-shaped as a whole, in *Kachuga*, *Cuora amboinensis*, *Cistoclemmys galbinifrons*, and *Mauremys japonica*. In *Cistoclemmys flavomarginata*, *Cuora* exclusive of *Cu. amboinensis*, *Geoemyda*, *Pyxidea*, *Rhinoclemmys annulata*, and *R. rubida*, the beak was unnotched and, slightly but distinctly hooked. We coded this character as: (0) notched and unhooked; (1) usually flat, but sometimes with a very weak notch or hook; (2) unnotched and distinctly hooked.

10. Ventromedial meeting of maxillae at bony beak of upper jaw

In most batagurid genera, the tip of the bony beak of the upper jaw was formed by the premaxilla, sometimes with very weak participation of the maxilla. In *Geoemyda*, the right and left maxillae were firmly connected ventromedially with each other, forming the beak. We coded this character as: (0) absent or sometimes present but very weak; (1) present.

11. Serration of upper labial margin of maxilla

Batagur, *Callagur*, *Hardella*, *Kachuga*, *Morenia*, and *Ocadia* of the batagurines had moderate to strong serration at the upper labial margin of the maxilla, whereas the other batagurines and most geoemydines did not have such serration. *Rhinoclemmys* species except for *R. rubida* had the serration weaker than in the above mentioned batagurines. We coded this character as: (0) absent; (1) present.

12. Size and shape of foramen palatinum posterius

The foramen palatinum posterius was small and round in *Cistoclemmys*, *Geoemyda*, and *Pyxidea*, as well as in the batagurines. On the other hand, the foramen was large, and elliptic or oval, about two times longer than wide, in the other geoemydines. We coded this character as: (0) large, and elliptic or oval; (1) small and round.

13. Anteromedial portion of upper triturating surface

In *Orlitia* and *Siebenrockiella*, and most geoemydines, the anteromedial portion of the upper triturating surface was narrower than the posterior portion, and the posteroventral portion of the premaxilla was excluded from the surface. In *Cistoclemmys*, *Geoemyda*, *Pyxidea*, *Rhinoclemmys annulata*, and *R. rubida*, the anteromedial portion of the upper triturating surface was expanded medially and broader than the posterior portion, and the posteroventral portion of the premaxillae participated in the triturating surface. This character state was usually observed in the batagurines except for *Orlitia* and *Siebenrockiella*. The posterior triturating surface was very narrow in *Cistoclemmys*, *Geoemyda*, *Pyxidea*, *R. annulata* and *R. rubida*, whereas it was broad in batagurines. We coded this character as: (0) narrower than posterior portion; (1) relatively narrow but expanding medially; (2) broad and expanding medially.

14. Contact between maxilla and vomer

A firm connection between the maxilla and vomer usually existed in most batagurid species. On the other hand, the contact was absent in *Cuora*, *Rhinoclemmys areolata*, *R. funerea*, *R. pulcherrima*, and *R. punctularia*, and it was very weak or sometimes absent in *Cistoclemmys flavomarginata*, *Mauremys*, and *Rhinoclemmys annulata*. We coded this character as: (0) present; (1) very weak, sometimes absent; (2) absent.

15. Position of foramen praepalatinum

In *Cistoclemmys flavomarginata*, *Cyclemys*, *Heosemys*, *Notochelys*, *Pyxidea*, and *Sacalia*, as well as in all batagurines, the foramen praepalatinum was located between the premaxilla and the vomer. In contrast, the foramen opened within the premaxilla in *Cuora*, *Geoemyda japonica*, *G. spengleri*, *Mauremys annamensis*, *M. mutica*, and *Rhinoclemmys* except for *R. annulata*. In the other geoemydines, the foramen was usually located near the suture between the premaxilla and vomer, or sometimes within the premaxilla. We coded this character as: (0) between premaxilla and vomer; (1) sometimes within premaxilla; (2) within premaxilla

16. Notch of prefrontal at posterior orbital margin

The prefrontal was usually shallowly notched at the posterior orbital margin in the *Cuora trifasciata* species-group and *Cu. mccordi*. Such a notch was absent in the other species. We coded this character as: (0) absent; (1) mostly present.

17. Contact between maxilla and pterygoid

The maxilla contacted the pterygoid in most batagurid species. On the other hand, such a contact was absent in *Cistoclemmys flavomarginata*, and was very weak or sometimes absent in *Geoemyda*. We coded this character as: (0) present; (1) very weak or sometimes absent; (2) absent.

18. Prootic participation in processus trochlearis oticum

Cyclemys, *Geoemyda silvatica*, *Heosemys*, *Mauremys*, *Notochelys* and *Sacalia*, as well as batagurines, had the processus trochlearis oticum mainly formed by the prootic. In *Cistoclemmys flavomarginata*, *Cuora*, *Geoemyda japonica*, *Melanochelys*, *Pyxidea*, and *Rhinoclemmys*, the prootic exposure was distinctly reduced, with the processus mainly formed by the quadrate. In the other geoemydines, the processus was formed by both prootic and quadrate. We coded this character as: (0) mainly by prootic; (1) by both prootic and quadrate; (2) mainly by quadrate.

19. Quadrate participation in canalis cavernosum

The quadrate participated in the canalis cavernosum in *Cuora*, while it usually did not in the remaining batagurids. We coded this character as: (0) absent; (1) usually present.

20. Hyoid apparatus

In most batagurid turtles, the hyoid was well ossified, and the second pair of branchial horns was slightly shorter than the first. On the other hand, the hyoid was weakly ossified (rather cartilaginous), and the second pair was much shorter than the first in *Cistoclemmys*, *Geoemyda*, *Pyxidea*, *Rhinoclemmys annulata*, and *R. rubida*. We coded this character as: (0) well ossified with second branchial horns slightly shorter than the first; (1) weakly ossified with second branchial horns much shorter than the first.

21. Plastral hinge

The plastral hinge was present in *Cistoclemmys*, *Cuora*, *Cyclemys*, *Notochelys*, and *Pyxidea*. They also possessed a well to extensively developed musulus testoscapularis and m. testoiliacus, which were associated with shell closure (Bramble, 1974). Among them, only *Cuora amboinensis* and *Cistoclemmys* closed both anterior and posterior plastral lobes without gaps between the carapace and plastron. We did not examine the condition of the muscles in *Batagur* and *Geoclemmys*, which lack a plastral hinge. The other species lacked a plastral hinge and m. testoscapularis and m. testoiliacus were poorly developed. We coded this character as: (0) absent; (1) present but impossible to close without gaps; (2) present and possible to close without gaps.

22. Anal notch of plastron

The plastron was distinctly notched posteromedially at the anal scutes in most batagurids. However, the notch was very shallow in *Cuora mccordi*, and absent in *Cistoclemmys*, and *Cuora amboinensis*. We coded this character as: (0) present; (1) present but very shallow; (2) absent.

23. Serration at posterior margin of carapace

Hieremys, *Siebenrockiella*, *Cyclemys*, *Geoemyda japonica*, *G. spengleri*, *Heosemys*, *Mauremys japonica*, *Notochelys*, and *Pyxidea* had strong serration at the posterior margin of carapace. In the other species, the serration was absent or weakly developed only in young individuals. We coded this character as: (0) absent, or weak and only in young individuals; (1) strong.

24. Longitudinal keels on carapace

Chinemys, *Geoclemys* and *Malayemys* of batagurines, and *Geoemyda*, *Melanochelys*, and *Pyxidea* of the geoemydines had three longitudinal rows of strong keels on the carapace with distinct ridges on the bony plates. In the other batagurid species, only the medial keel was usually developed: the lateral keels were absent or, if present, very weak without distinct ridges on the bony plates. We coded this character as: (0) weak, usually in a single row; (1) prominent, in three rows.

25. Anterior part of neural bones

In *Hieremys* of the batagurines and the geoemydines exclusive of *Mauremys*, *Sacalia*, and *Notochelys*, the anterior neurals were usually hexagonal with posterior sides shorter than anterior sides. In the other species, the neurals were also usually hexagonal but the anterior sides were shorter than the posterior sides. We coded this character as: (0) short-sided anteriorly; (1) short-sided posteriorly.

26. Sutures between lateral sides of seventh and eighth pleurals

In most batagurids, the seventh and eighth pleurals on each side were separated by the eighth neural and the anterior suprapygal. In *Cistoclemmys*, *Cuora amboinensis*, and *Rhinoclemmys rubida*, the eighth neural and the anterior suprapygal were usually absent, and the lateral sides of seventh and eighth pleurals were sutured with each other. We coded this character as: (0) absent; (1) present.

27. Cloacal bursae

The cloacal bursae were present in most batagurids, while it was much reduced or completely lost in *Cistoclemmys*, *Pyxidea*, and *Geoemyda*. We could not examine the condition of this character in *Batagur* and *Geoclemmys*. We coded this character as: (0) present; (1) much reduced or lost.

28. Axillary plastral buttress

In *Chinemys*, *Hieremys*, and *Morenia* of the batagurines, and in most geoemydines having no plastral hinge, the dorsal portion of the axillary plastral buttress was connected around the portion between the peripherals and the costals, and the connected portion was distinctly concave. In all geoemydines having a plastral hinge (*Cyclemys*, *Notochelys*, *Cuora*, *Cistoclemmys*, and *Pyxidea*: see above) the dorsal portion of the buttress was very short and connected to the peripherals, and the connected portion was not or only scarcely concave. In *Mauremys annamensis* of the geoemydines, and in *Geoclemys*, *Malayemys*, *Orlitia*, and *Siebenrockiella* of the batagurines, the dorsal portion of the buttress was long, expanded, and strongly connected to the costals, and the connected portion was distinctly concave. In the other batagurines, the buttress was much more developed than in *M. annamensis*, *Malayemys*, *Orlitia*, and *Siebenrockiella*, and was very strongly connected to the costals, almost reaching the ribs. We coded this character as: (0) very strongly connected to costals, almost reaching ribs; (1) strongly connected to costals; (2) connected around the portion between peripherals and costals; (3) weakly connected to the peripherals.

29. Inguinal plastral buttress

In *Chinemys*, *Malayemys*, *Morenia*, and *Orlitia* of the batagurines, and most geoemydines, the dorsal portion of inguinal plastral buttress was connected around the suture between the peripherals and the costals, and the connected portion was distinctly concave. In *Mauremys annamensis*, *Geoclemys*, and *Siebenrockiella*, the dorsal portion of the buttress was long, expanded, and strongly connected to the costals, and the connected portion was distinctly concave. In *Hieremys*, *Cistoclemmys*, *Cuora*, and *Pyxidea*, the dorsal portion was very short, and the connected portion was not concave. In the other batagurines, the buttress was strongly developed, almost touching the rib. We coded this character in the same way as Character 28.

30. Internal choanae

Parsons (1960) examined the structure of the choanae for most batagurids. We followed his terminology for the structure. Our examination revealed that *Chinemys*, *Hieremys*, *Ocadia*, *Orlitia*, and *Siebenrockiella* of batagurines, and most geoemydines had the internal choanae with a flap or a ridge. Some specimens of *Mauremys mutica*, *Rhinoclemmys pulcherrima*, and *Cistoclemmys flavomarginata* had the internal choanae with a very weak flap, resembling a ridge. In *Mauremys rivulata*, *M. mutica*, *Melanochelys*, and *Rhinoclemmys pulcherrima*, some specimens had a flap and others had a ridge. The internal choanae had a small papilla in *Callagur*, *Hardella*, *Kachuga*, *Morenia*, 2/4 of *Notochelys*, *Cyclemys*, *Rhinoclemmys funerea*, and *R. punctularia*. The internal choanae lacked a papilla, flap, or ridge in *Malayemys* and *Heosemys*. We could not examine this character state in *Batagur* and *Geoclemys*. We coded this character as: (0) without papilla, flap, or ridge; (1) with flap or ridge; (2) usually with papilla.

31. Skin of posterior head

The skin of the posterior head was smooth in most geoemydines, but subdivided into small scales in *Cyclemys*, *Geoemyda silvatica*, *Heosemys*, *Melanochelys*, *Notochelys*, and *Pyxidea* of the geoemydines, as well as in the batagurines. We coded this character as: (0) subdivided into small scales; (1) smooth.

32. Radiated markings on plastron

Radiated markings were observed on each plastral scute in *Cyclemys* and *Heosemys*. We coded this character as: (0) absent; (1) present.

33. Entoplastron

In turtle taxonomy, many authors paid attention to whether the entoplastron was intersected by the humero-pectoral seam (e.g., Boulenger, 1889; Smith, 1931; Bourret, 1941, Pritchard, 1979; Ernst and Barbour, 1989). Our examination revealed that among batagurines, the state of this character was variable, seemingly reflecting the phylogeny (Hirayama, 1984). On the other hand, the entoplastron was consistently intersected by the seam in all the geoemydines but *Geoemyda silvatica*. Therefore, the absence or presence of the intersection was not informative for the inference of geoemydine phylogeny. In the specimens of *G. silvatica* examined by us, the entoplastron was intersected by the seam near the posterior rim (4/5) or overlapped by the seam at the posterior rim (1/5). We thus considered that the absence of the intersection represented rather minor individual variation in this species, although Moll et al. (1986) reported the absence of intersection of entoplastron by the humero-pectoral seam in this species.

We also examined whether the entoplastron was intersected by the gulo-humeral seam. In most batagurid species, the entoplastron was intersected by this seam or barely separated from it (1/10 of *M. mutica* and 2/9 of *Sacalia*), but in *Geoemyda* the entoplastron was in a separate location posterior to the gulo-humeral seam. We coded the character as: (0) entoplastron mostly intersected by gulo-humeral seam; (1) entoplastron separated from gulo-humeral seam.

34. Antero-dorsal portion of iliac blade

In most batagurids, the antero-dorsal portion of the iliac blade was about as long as its postero-dorsal portion (Fig. 1b, e–j). Only in *Cistoclemmys*, the antero-dorsal portion was about two times longer than the postero-dorsal portion (Fig. 1c, d). We coded this character as: (0) as long as its postero-dorsal portion; (1) two times longer than its postero-dorsal portion.

35. Small foramen on ventral maxilla

Rhinoclemmys funerea and *R. punctularia* usually had a very small foramen on the ventromedial portion of the maxilla. Such a foramen was absent in the remaining batagurids. We coded this character as: (0) absent; (1) usually present.

RESULTS

All characters examined were informative for the inference of relationships among the 26 geoemydine OTUs.

Three equally parsimonious trees (tree length=107; consistency index (CI)=0.449; retention index=0.762; rescaled consistency index=0.342) were detected. *Mauremys japonica*, the *M. leprosa* species-group, and the *M. annamensis–M. mutica* clade were trifurcated in all trees. These trees differed from each other in topology involving *Rhinoclemmys annulata*, *R. rubida*, and the *Geoemyda–Cistoclemmys–Pyxidea* clade. In the strict consensus tree (Fig. 3), therefore, their relationships were also expressed as trifurcated.

The consistency index for these data was low (CI=0.449). However, this index is known to be highly negatively correlated with numbers of OTUs included in an analysis (Sanderson and Donoghue, 1989; Klassen et al., 1991). Our analysis involved a relatively large number of OTUs (28), and the value, while being similar to that in a

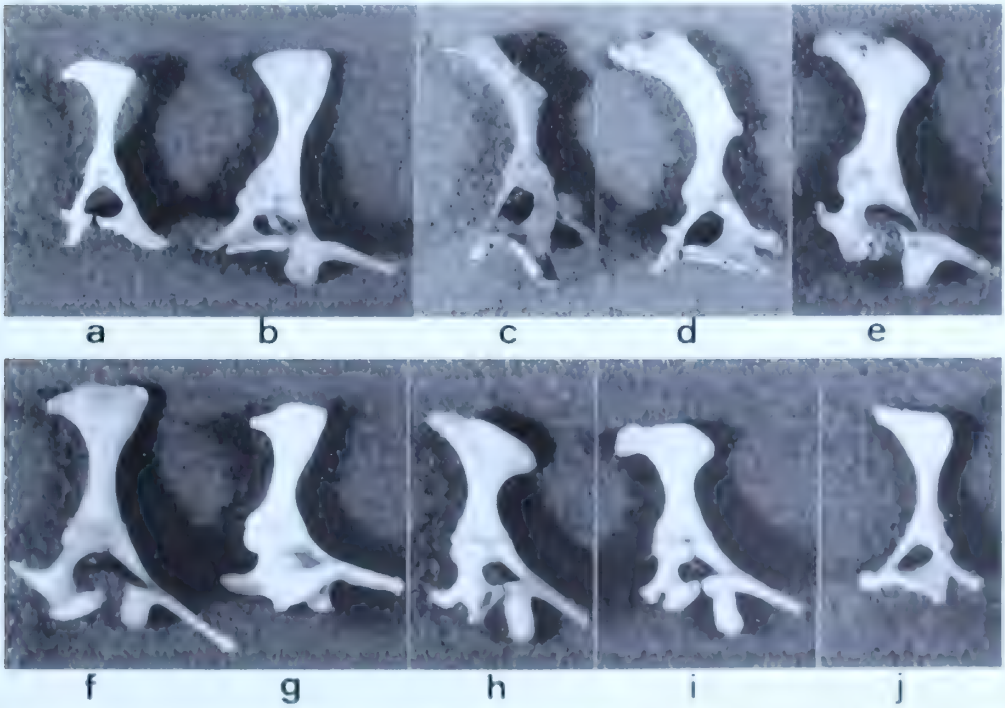


FIG. 1. Medial views of pelvic girdles. Batagurinae: (a) *Chinemys reevesii*, (b) *Siebenrockiella crassicollis*; Geoemydinae: (c) *Cistoclemmys flavomarginata flavomarginata*, (d) *C. galbinifrons*, (e) *Pyxidea mouhotii*, (f) *Mauremys japonica*, (g) *M. mutica mutica*, (h) *Cuora amboinensis*, (i) *Rhinoclemmys pulcherrima manni*, (j) *Geoemyda japonica*.

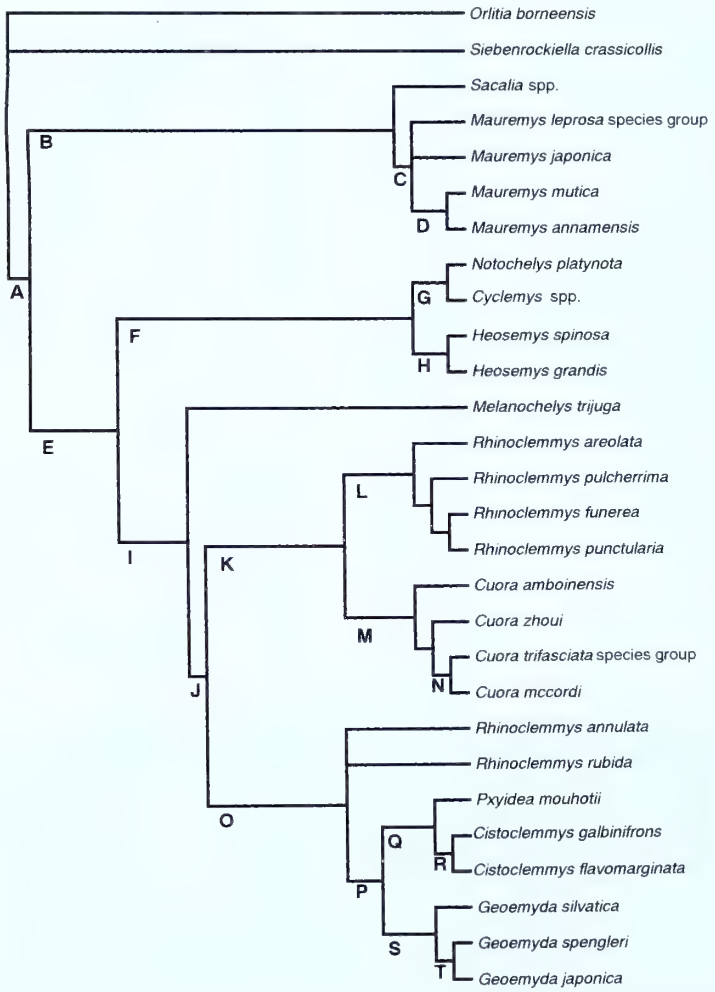


FIG. 3. Strict consensus cladogram of the subfamily Geoemydinae and its relatives.

previous empirical study dealing with a comparable number of OTUs (0.451 for 28 taxa: Sanderson and Donoghue, 1989), was much greater than that expected for random data (0.131 for 28 taxa; Klassen et al., 1991). Thus, we consider the monophyletic groups indicated in our results significantly realistic.

At the root of the Geoemydinae (Stem A), characters 1–27 and 31–35 were in the "0" state, whereas the characters 28–30 were "1". We regarded these character states as ancestral within the Geoemydinae. Putative changes of character states in each major stem are given in Table 3.

Supposing that all character states in the outgroup represent primitive conditions, the monophyly of Geoemydinae (Stem A) was supported by three character states: character 1 (state 0), presence of frontal exposure on orbital rim; 12 (0), large foramen palatinum posterius with elliptic or oval shape; and 28 (2), axillary plastral buttress connected around the portion between peripherals and costals.

One synapomorphic character state, 31 (1) (smooth skin of posterior head), supported the *Mauremys–Sacalia* clade (Stem B), which we henceforth call the *Mauremys* group.

TABLE 3. Putative changes of character states in major stems of Geoemydinae. Letters denoting stems refer to those in Fig. 3.

Stem	Character	Change
A	1	1 to 0
	12	1 to 0
	28	1 to 2
B	31	0 to 1
C	14	0 to 1
	15	0 to 1
D	15	1 to 2
E	7	0 to 1
	8	0 to 1
	25	0 to 1
F	23	0 to 1
	32	0 to 1
G	21	0 to 1
	28	2 to 3
	30	1 to 2
H	6	0 to 1
	30	1 to 0
I	15	0 to 1
	18	0 to 2
J	9	0 to 1
	31	0 to 1
K	11	0 to 1
	14	0 to 2
	15	1 to 2
L	8	1 to 0
	9	1 to 0
M	19	0 to 1
	21	0 to 1
	28	2 to 3
	29	2 to 3
N	16	0 to 1
O	9	1 to 2
	13	0 to 1
	20	0 to 1
P	2	0 to 2
	3	0 to 1
	4	0 to 1
	5	0 to 1
	12	0 to 1
	24	0 to 1
	27	0 to 1
Q	15	1 to 0
	21	0 to 1
	28	2 to 3
	29	2 to 3
R	21	1 to 2
	22	0 to 2
	24	1 to 0
	26	0 to 1
	34	0 to 1
S	8	1 to 0
	10	0 to 1
	17	0 to 1
	18	2 to 1
	33	0 to 1
T	3	1 to 2
	15	1 to 2
	23	0 to 1

The genus *Mauremys* (Stem C) was supported by two character states: 14 (1), maxilla sometimes contacting vomer; and 15 (1), foramen praepalatinum sometimes opening within premaxilla. Autapomorphy of *Sacalia* was not detected. The *M. annamensis–M. mutica* clade (Stem D) was supported by 15 (2), foramen praepalatinum opening within premaxilla.

Stem E, henceforth referred to as the *Geoemyda* group, was supported by three synapomorphic character states: 7 (1), absence of the squamosal-quadratojugal contact; 8 (1), absence of the jugal-quadratojugal contact; and 25 (1), anterior neurals with shorter posterior sides. The former two character states are seemingly associated with the reduction of the quadratojugal. The state of the third character was constant within the group except for *Notochelys*.

The *Notochelys-Cyclemys-Heosemys* clade (Stem F) was supported by two synapomorphic character states: 23 (1), strong serration at posterior margin of carapace; and 32 (1), presence of radiated markings on plastron. The *Notochelys–Cyclemys* clade (Stem G) was supported by three synapomorphic character states: 21 (1), plastral hinge not closing without gap; 28 (3), very weak axillary plastral buttress; and 30 (2), internal choanae usually with a small papillia. Monophyly of *Heosemys* (Stem H) was supported by two synapomorphic character states: 6 (1), loss of quadratojugal; and 30 (0), internal choanae without ridge, flap, or papillae. The latter was unique among the geoemydines. *Heosemys* was the sister group of the *Notochelys–Cyclemys* clade.

Stem I was supported by two synapomorphic character states: 15 (1), foramen praepalatinum sometimes opening within premaxilla; and 18 (2), processus trochlearis oticum mainly formed by the quadrate. Stem J was supported by two synapomorphic character states, 9 (1: usually flat jaw beak) and 31 (1: smooth posterior head skin), whereas *M. trijuga* had one autoapomorphic character state, 24 (1: three prominent keels).

Rhinoclemmys was shown to be polyphyletic, consisting of two groups, *Rhinoclemmys* A (*areolata*, *funerea*, *punctularia* and *pulcherrima*) and *Rhinoclemmys* B (*annulata* and *rubida*). The *Rhinoclemmys* A–*Cuora* clade (Stem K) was supported by three character states: 11 (1), very weak serration of upper labial margin; 14 (2), absence of the maxilla–vomer contact; and 15 (2), location of foramen praepalatinum within premaxilla. Within this clade, monophyly of the *Rhinoclemmys* A (Stem L) was supported by two character states, 8 (0: the jugal–quadratojugal contact) and 9 (0: notched and hooked beak of upper jaw), whereas the *R. funerea–R. punctularia* clade was supported by a unique synapomorphic character state 35 (1: usual presence of small foramen on ventral maxilla). *Cuora* (Stem M) was further supported by four synapomorphic character states: 19 (1), quadrate participation into canalis cavernosum; 21 (1), presence of both plastral hinge and gap between closed shells; 28 (3), very weak axillary plastral buttress; and 29 (3), very weak inguinal plastral buttress. The *Cuora trifasciata* species group–*Cu. mccordi* clade (Stem N) was supported by a unique character state: 16 (1), prefrontal notched at posterior orbital margin.

Although there were no synapomorphies uniting the *Rhinoclemmys* B, the *Rhinoclemmys* B–*Cistoclemmys–Pyxidea–Geoemyda* clade (Stem O) was supported by three character states: 9 (2), unnotched and hooked upper jaw beak; 13 (1), anteromedial portion of triturating surface expanding medially; and 20 (1), reduction of hyoid apparatus.

Monophyly of the *Cistoclemmys–Pyxidea–Geoemyda* clade (Stem P) was supported by seven synapomorphic character states, 3 (1), foramen nervi vidiani located anteroventrally or laterally to anteroventral part of processus inferior parietalis; 4 (1), medial process of jugal with small tip; 27 (1), strong reduction or loss of cloacal bursae; 2 (2), strong ventral convergence of cranial cavity; 5 (1), usual absence of the jugal–pterygoid contact; 12 (1), small and round foramen palatinum posterius; and 24 (1), three prominent keels on carapace. The former three were unique among geoemydines. The *Cistoclemmys–Pyxidea* clade (Stem Q) was monophyletic on the basis of four character states: 15 (0), foramen praepalatinum located between premaxilla and vomer; 21 (1), presence of plastral hinge; 28 (3), very weak axillary plastral buttress; and 29 (3), very weak inguinal plastral buttress. Validity of *Cistoclemmys* (Stem R) was supported by five synapomorphic character states: 21 (2), plastral hinge closing shell without gap; 22 (2), absence of anal notch of plastron; 24 (0), usual absence of lateral keels on carapace; 26 (1), presence of sutures between lateral sides of seventh and eighth pleurals; and 34 (1), very long anterodorsal portion of iliac blade (unique in geoemydines, Fig. 1c, d).

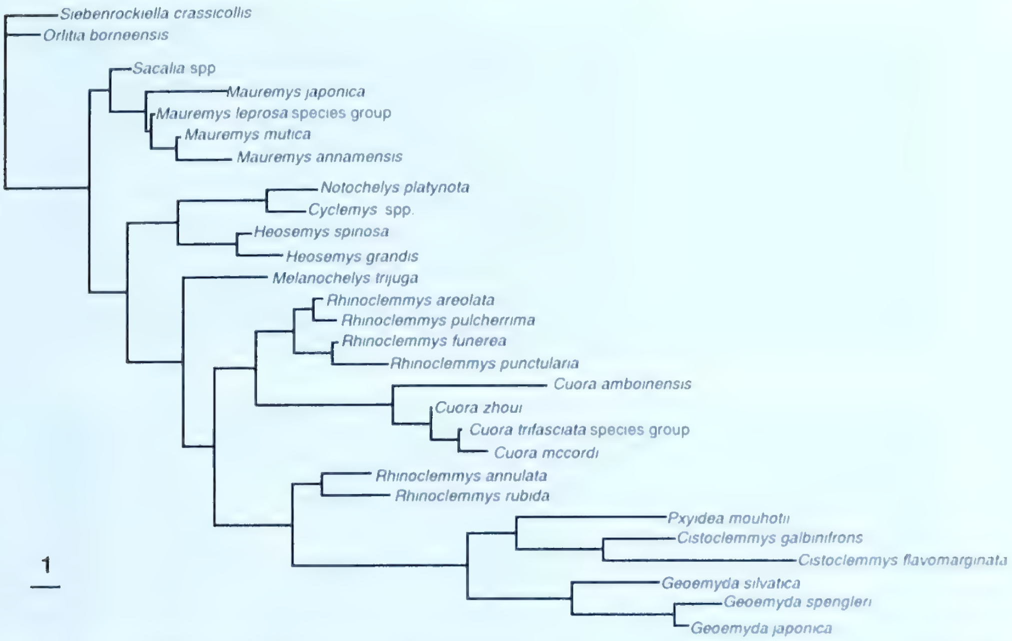


FIG. 4. Phylogram by neighbor-joining method of the subfamily Geoemydinae and its relatives.

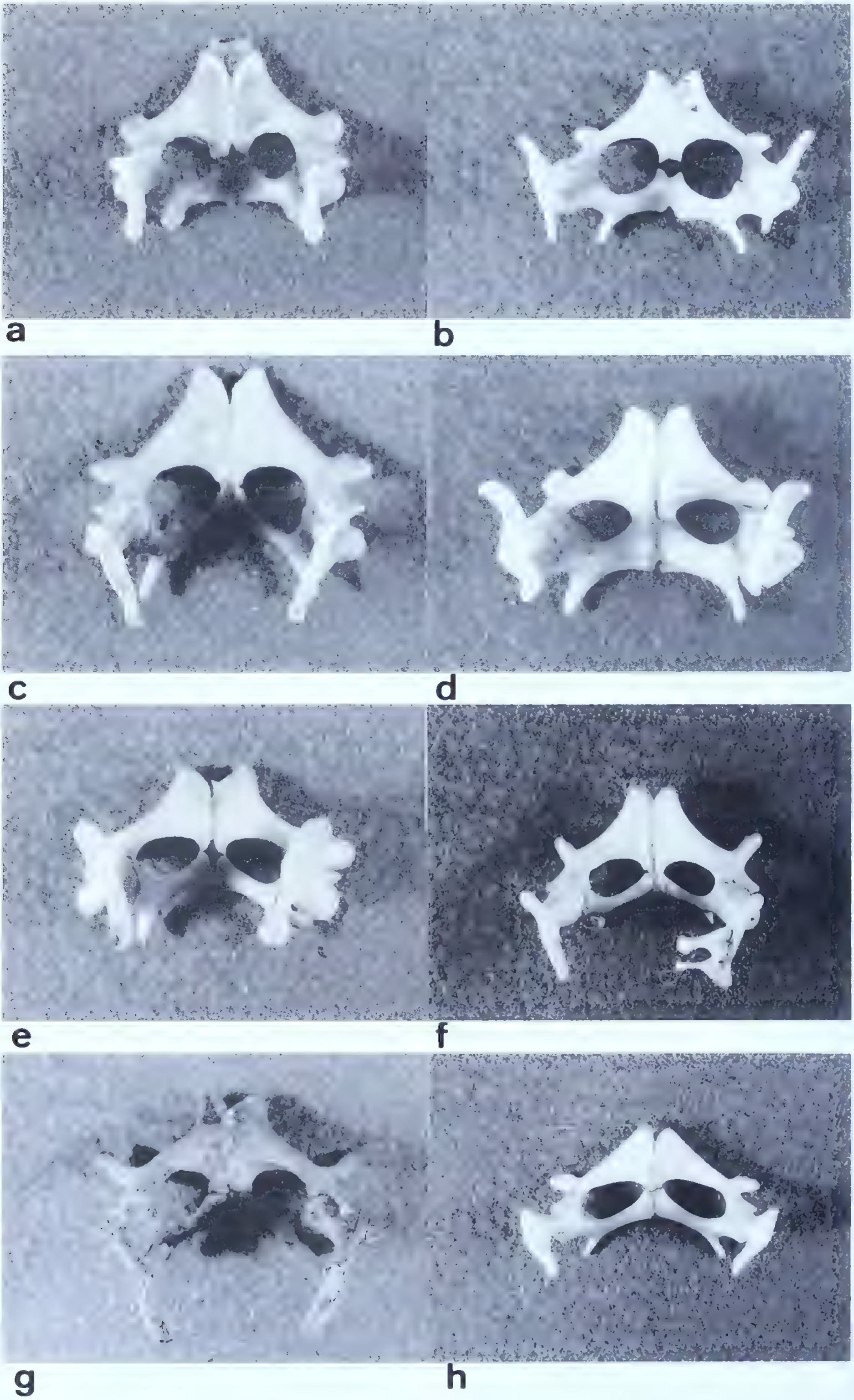
Pyxidea had two autoapomorphic character states: 23 (1), strong serration of posterior carapace margin; and 31 (1), posterior head skin subdivided into small scales. Monophyly of *Geoemyda* (Stem S) was supported by five character states: 10 (1), ventromedial meeting of maxillae; 33 (1), entoplastron separated from gulo-humeral seam; 8 (0), presence of the jugal-quadratojugal contact; 17 (1), very weak maxilla-pterygoid contact; and 18 (1) processus trochlearis oticum formed with prootic and quadrate. The former two character states were unique in the Bataguridae. In addition, the *G. spengleri–G. japonica* clade (Stem T) was supported by three synapomorphic character states: 3 (2), foramen nervi vidiani sometimes located on pterygoid; 15 (2), foramen praepalatinum opening within premaxilla; and 23 (1), strong serration at posterior carapace margin. The first was unique within the Bataguridae.

The phylogram, obtained by the neighbor-joining (NJ) method using an absolute distance matrix is shown in Fig. 4. This phylogram is largely identical in topology with the strict consensus tree from the parsimony analysis (Fig. 3), and thus is interpreted as supporting the validity of the latter. One of the differences between the strict consensus tree and the NJ phylogram lay in the relationships within the *Rhinoclemmys* A. In the latter tree, *R. pulcherrima* was united not with the *R. funerea-R. punctularia* group but with *R. areolata*. Another difference was in the relationships among *R. annulata*, *R. rubida*, and the *Cistoclemmys-Pyxidea-Geoemyda* group. In the NJ phylogram, *R. annulata* and *R. rubida* were united, collectively constituting the first outgroup to the *Cistoclemmys-Pyxidea-Geoemyda* group.

DISCUSSION

Monophyly of the Bataguridae

Based on the analyses of morphological and mithochondrial DNA characters for major testudine taxa including one batagurine, one geoemydine, three emydid, and one testudinid genera, Shaffer et al. (1997)


a
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argued that both Bataguridae and Emydidae are probably monophyletic, and that the latter is the sister group of a clade consisting of the former and the Testudinidae. Recently we also found states of two morphological characters (i.e., well developed foramina of axillary and inguinal musk ducts with penetration into the breast chamber, and anterolaterally flared iliac blade [Fig. 2]) that seemingly lend further support to the monophyly of the Bataguridae (Yasukawa, 1997; Hirayama, Yasukawa and Aoki, unpublished data), because they occur in most batagurid turtles but not in the Emydidae or Testudinidae. In our examinations, the former character state occurred in all batagurids exclusive of *Morenia*. Musk ducts and indistinct musk foramina also exist in some non-testudinoid families of turtles, such as the Chelydridae, Chelydae, Kinosternidae, and Platysternidae, and their occurrence is thus likely to represent primitive conditions in the Testudines (Gaffney and Meylan, 1988). However, development of musk duct foramina to the point of reaching the breast chamber is a condition unique to the Bataguridae (Hirayama, Yasukawa and Aoki, unpublished data). On the other hand, an anterolaterally flared iliac blade was observed in all batagurid, but not in other turtles including those belonging to non-testudinoid families. We excluded these characters from our analyses, since these are obviously not informative for the inference of relationships among the geoemydines. However, the states of these characters are possibly synapomorphs of the Bataguridae. Thus, in the sections below we take an a priori assumption of the batagurid monophyly.

Monophyly of the Geoemydinae

McDowell (1964) referred to two character states as discriminating Geoemydinae from Batagurinae—narrow and flat triturating surfaces, and reduction or absence of the quadratojugal. The former probably represents a primitive condition of the Testudinoidea (Hirayama, 1984; Gaffney and Meylan, 1988). In this study, the latter condition is observed in the *Geoemyda* group exclusive of *G. spengleri*. Because the *Mauremys* group had a relatively large quadratojugal as in *Kachuga*, *Ocadia*, *Odsrlitia*, and *Siebenrockiella*, it seems to be more appropriate to consider the reduction or absence of the quadratojugal as a characteristic of the *Geoemyda* group rather than as a synapomorph of the Geoemydinae.

Hirayama (1984) considered that the subdivision of the foramen nervi trigeminalis, observed in more than 40% of the specimens examined for each geoemydine species, is a synapomorph of the Geoemydinae besides the loss or reduction of the quadratojugal. However, Gaffney and Meylan (1988) were concerned about such an inconsistent occurrence of the former character state, and argued that its use at this level was dubious. Our additional observations also confirmed the remarkable variability of this character at the intraspecific level (Yasukawa, unpublished observation). Therefore, we did not include this character in our phylogenetic analyses. Although Gaffney and Meylan (1988) regarded the loss or reduction of the quadratojugal as "relatively consistent" in geoemydines, this character state turned out to be confined to the *Geoemyda* group alone (see above).

Our analyses yielded states of three characters as possibly supporting monophyly of

FIG. 2. Dorsal views of pelvic girdles. Emydidae: (a) *Trachemys scripta elegans*; Batagurinae: (b) *Chinemys reevesii*; Geoemydinae: (c) *Mauremys japonica*, (d) *M. mutica mutica*, (e) *Cuora trifasciata*, (f) *Rhinoclemmys pulcherrima manni*, (g) *Cistoclemmys flavomarginata flavomarginata*, (h) *Geoemyda japonica*.

TABLE 4. Classifications of geoemydine turtles of the family Bataguridae, derived from results of this study. "*": species not examined; "?": species of dubious generic allocation.

Testudinoidea		
Testudinidae		
Emydidae		
Bataguridae		
Batagurinae		
Geoemydinae		
Geoemyda group	11 genera	34 species
Genus *Chelopus*		2 species
C. annulatus		
C. rubidus		
Genus *Cistoclemmys*		2 species
C. flavomarginata		
C. galbinifrons		
Genus *Cuora*		7 species
C. amboinensis		
C. aurocapitata		
C. mccordi		
C. pani		
C. trifasciata		
*C. yunnanensis**		
C. zhoui		
Genus *Cyclemys*		4 species
C. dentata		
C. oldhamii		
C. pulchristriata		
C. tcheponensis		
Genus *Geoemyda*		4 species
G. japonica		
G. silvatica		
G. spengleri		
?*G. leytensis**		
Genus *Heosemys*		3 species
*H. depressa**		
H. grandis		
H. spinosa		
Genus *Leucocephalon*		1 species
*L. yuwonoi**		
Genus *Melanochelys*		2 species
*M. tricarinata**		
M. trijuga		
Genus *Notochelys*		1 species
N. platynota		
Genus *Pyxidea*		1 species
P. mouhotii		
Genus *Rhinoclemmys*		7 species
R. areolata		
*R. diademata**		
R. funerea		
*R. melanosterna**		
*R. nasuta**		
R. pulcherrima		
R. punctularia		
Mauremys group	2 genera	11 species
Genus *Mauremys*		8 species
M. annamensis		
M. caspica		
*M. iversoni**		
M. japonica		
M. leprosa		
M. mutica		
?*M. pritchardi**		
M. rivulata		
Genus *Sacalia*		3 species
S. bealei		
*S. pseudocellata**		
S. quadriocellata		

the Geoemydinae: 1 (0), presence of frontal exposure on orbital rim; 12 (0), large and elongated foramen palatinum posterius; and 28 (2), axillary plastral buttress connected around the portion between peripherals and costals. Of these, however, the first and the third were also observed in various species of the Batagurinae (Table 1), Emydidae, and Testudinidae (Gaffney, 1979; Yasukawa and Hirayama, unpublished data), and thus seemingly represent primitive conditions of the Testudinoidea. The second was observed in all geoemydines except for the *Cistoclemmys–Pyxidea–Geoemyda* clade and thus may be a synapomorph of the subfamily. However, we failed to determine whether this state represents a derived or primitive condition, because the character is highly variable within the Testudinoidea (Gaffney and Meylan, 1988; Yasukawa, unpublished data), and is also seen in Mesozoic primitive testudinoids such as *Mongolemys* (Hirayama, unpublished data).

There are no synapomorphs clearly supporting the monophyly of Geoemydinae. The character states shared by most members of this subfamily largely represent primitive conditions in the Bataguridae (Hirayama, 1984; Table 1). This may suggest the paraphyletic nature of the Geoemydinae as an assemblage of primitive stocks of the Bataguridae. Nevertheless, because we could also detect no synapomorphs supporting sister group relationship with the Batagurinae of any particular clade within the Geoemydinae, this problem remains to be readdressed on the basis of additional data. In consideration of taxonomic stability, we propose to temporarily retain the recognition of the subfamily Geoemydinae as a "metataxon", for which no character evidence supports or negates the monophyly (Gauthier et al., 1988).

Our analyses revealed that the Geoemydinae consists of the *Mauremys* and the *Geoemyda* groups. In the following subsections, we discuss the phylogeny within each of these groups. We also propose several modifications to the classification by David (1994) (Table 4) on the basis of evolutionary relationships revealed in this study.

Mauremys *group*

The results of this study may support the monophyly of the *Mauremys* group as consisting of *Mauremys* and *Sacalia* with one synapomorph (31 [1], smooth skin of the posterior head). Even so, however, this character state was also observed in Stem J except for *Geoemyda silvatica* and *Pyxidea*. This suggests that 31 (1) has evolved more than one time in the Geoemydinae, and reduces it value as evidence for monophyly of the *Mauremys* group. Other character states exhibited by *Mauremys* and *Sacalia* seem to mostly represent primitive conditions of the Geoemydinae (Table 1), or of the whole family Bataguridae (Hirayama, 1984). Thus, the *Mauremys* group may be referred to as the primitive stock of this family.

Monophyly of the genus *Mauremys* was supported by two character states, 14 (1) and 15 (1), while *Sacalia* exhibited no autapomorphs in our parsimony analyses. McDowell (1964) suggested that *Sacalia* is discriminated from *Mauremys* in four character states. Of these, however, only one (a few very large scutes without intervening granules on forearms) is a possible synapomorph of the *Sacalia* species. Although we did not employ scutellation of the forearm in the phylogenetic analyses due to its extensive intrageneric variation in the *Geoemyda* group, we confirmed the unique state mentioned above in this character in *Sacalia*. Considering it as an autapomorph of *Sacalia*, we regard both *Mauremys* and *Sacalia* as valid.

Geoemyda *group*

In our results, the monophyly of the *Geoemyda* group is supported by three character states, absence of the squamosal-quadratojugal contact (7 [1]), absence of the jugal–quadratojugal contact (8 [1]), and anterior neurals with shorter posterior sides (25 [1]). Of these, 25 (1) was constant within the *Geoemyda* group exclusive of *Notochelys*, in which the anterior neurals had shorter anterior sides like those in the *Mauremys* group and most batagurines. Hirayama (1984) depicted a sister group relationship between *Sacalia* and *Notochelys* as supported by jugal-parietal contact, and distinctly widened first vertebral which sometimes reaches the second marginal. The present study revealed that the jugal is close to the parietal but separated from it in *Notochelys*, while the two make weak contact in *Sacalia*. The first vertebral was widened and usually reached the second marginal in *Mauremys japonica*, the *M. leprosa* species-group, *Sacalia*, and sometimes in *Notochelys*, *M. annamensis*, *M. mutica*, the *Cuora trifasciata* species-group, *Cu. zhoui*, and *Melanochelys*. Thus, it is obviously inadequate to use these characters to unite *Sacalia* and *Notochelys*. In the present analyses, the *Notochelys–Cyclemys* clade is supported by three synapomorphic character states, and we thus consider this relationship to be far more likely than the *Notochelys–Sacalia* monophyly.

Except for the position of *Notochelys* (see above), our results largely support Hirayama's (1984) hypothesis in that they also suggest: polyphyly of *Cuora* (sensu lato) and validity of *Cistoclemmys*; close relationships among *Cistoclemmys*, *Pyxidea* and *Geoemyda*; and polyphyly of *Rhinoclemmys*. Sites et al. (1984), on the basis of the analysis of allozymic variation among 22 batagurid species, suggested that *Cuora* and *Cistoclemmys* are most closely related to each other. This has been one of the major reasons to regard *Cistoclemmys* as a junior synonym of *Cuora* (e.g., Ernst and Barbour, 1989; McCord and Iverson, 1991; Iverson, 1992). However, Sites et al. (1984) examined very small numbers of individuals for most species. The number of loci examined in that study was also very small. Our results showed that *Cistoclemmys* is related more closely to *Pyxidea* and *Geoemyda* than to *Cuora*, although *Cistoclemmys*, *Pyxidea*, and *Geoemyda* are well differentiated from each other (Fig. 4). Based on the external comparisons of subspecies of *Cistoclemmys galbinifrons* and *Pyxidea*, Fritz and Obst (1997) concluded that *Ci. g. serrata* is a distinct species which narrows the gap between *Cuora* (sensu lato) and *Pyxidea*. They doubted the validity of both *Cistoclemmys* and *Pyxidea*. However, the number of specimens of *Ci. g. serrata* examined by them was very small. Moreover, they did not examine internal characters, in which we did find a number of remarkable differences among *Cistoclemmys*, *Cuora*, and *Pyxidea*. We thus consider the three genera to be valid.

The results of this study confirmed the polyphyly of *Rhinoclemmys* as proposed by Hirayama (1984), because they divided *Rhinoclemmys* into two groups belonging to different clades. Of these, *Rhinoclemmys* A consisted of two aquatic species, *punctularia* and *funerea*, and two terrestrial species, *areolata* and *pulcherrima*. *Rhinoclemmys* B, on the other hand, consisted of two terrestrial species with unnotched, hooked beaks, *annulata* and *rubida*, that were not united into a monophyletic group by the parsimonious analysis, but did constitute a single unit in the NJ phylogram. We thus discuss the relationship of these two *Rhinoclemmys* B species below.

Based exclusively on the coloration and the relative depth of the shell, Ernst (1978) demonstrated that *Rhinoclemmys* (as *Callopsis*) can be divided into three groups, the *pulcherrima–rubida* group, *punctularia* group (as consisting of *R. punctularia*, *R. annulata*, *R. diademata*, *R. funerea*, *R. melanosterna*, and *R. nasuta*), and *R. areolata*. This grouping substantially contradicts our results, especially in the positions of *R. annulata*, *R. pulcherrima*, and *R. rubida*. Considering the remarkable intersubspecific variation in those characters in both *R. pulcherrima* and *R. rubida* (Pritchard and Trebbau, 1984; Ernst and Barbour, 1989), the grouping proposed by Ernst (1978) is dubious.

Sites et al. (1981), in the assessment of allozymic variation among *R. areolata*, *R. funerea*, *R. pulcherrima*, *R. punctularia*, and *R. rubida*, suggested close affinity among *R. areolata*, *R. punctularia*, and *R. funerea*, and distinct differentiation among the *areolata–punctularia–funerea* assemblage, *R. pulcherrima*, and *R. rubida*. Our results are in accord with those of Sites et al. (1981) in showing close relationship among *R. punctularia*, *R. funerea*, and *R. areolata*, but contradict the latter in showing the sole divergence of *R. rubida*. Because the results of Sites et al. (1981) were derived from analyses of a relatively small number of loci for very small samples, we consider that our results more successfully reflect the phylogeny of *Rhinoclemmys*.

The type species of *Rhinoclemmys* Fitzinger, 1835, is *Testudo dorsata* Schoepff, 1801, which is a junior synonym of *R. punctularia* (see Iverson, 1992). Therefore, *punctularia* and its relatives (*areolata*, *funerea*, and *pulcherrima*) should be retained under *Rhinoclemmys*, while *rubida* (type species of *Chelopus*) and *annulata* should be moved to the resurrected genus *Chelopus* Cope, 1870, which was formerly regarded as a junior synonym of *Rhinoclemmys* (see Wermuth and Mertens, 1977).

Parallel evolution of plastral hinge

Within the Bataguridae, a kinetic plastron equipped with a hinge between the pectoral and abdominal was observed in five geoemydine genera, *Cistoclemmys*, *Cuora*, *Cyclemys*, *Notochelys*, and *Pyxidea*. Bramble (1974) demonstrated distinct differences in shell-closing mechanism between emydid box turtles and batagurid box turtles. He argued that in the Geoemydinae the plastral hinge had evolved two times, in the common ancestor of *Cistoclemmys*, *Cuora*, *Cyclemys*, and *Pyxidea*, and in *Notochelys*. In addition, he noted that *Heosemys* had a more developed musclus testoscapularis (a muscle element associated with the closure of the anterior plastral lobe in the batagurid box turtles: Bramble, 1974) than the other geoemydines lacking a plastral hinge. He thus suspected the derivation of *Cistoclemmys*, *Cuora*, *Cyclemys*, and *Pyxidea* from a common *Heosemys*-like ancestor, and, on the basis of this consideration, united the five genera in the *Heosemys* group. However, our study showed no distinct differences in the degree of development of m. testoscapularis between *Heosemys* and other "non-box" geoemydines (character 21). Additionally, we did not find any character to support monophyly of the *Heosemys* group, or of an assemblage of *Cistoclemmys*, *Cuora*, *Cyclemys*, and *Pyxidea*. We thus consider Bramble's (1974) grouping as unnatural.

Bramble (1974) also suggested that plastral kinesis of *Notochelys* arose apart from the *Heosemys* group. But he hardly mentioned the difference in shell-closing mechanism between *Notochelys* and the other batagurid box turtles. In adult living specimens, both anterior and posterior plastral lobes of *Notochelys* are as movable as those of *Cyclemys* but less movable than those of *Cistoclemmys*, *Cuora*, and *Pyxidea* (Yasukawa, unpublished data). Both *Cyclemys* and *Notochelys* have no hinge in juveniles, while the other three genera have a weak hinge even in juveniles (Yasukawa, unpublished data). In contrast, the very long anterodorsal portion of the iliac blade, a condition unique to the batagurids (character state 34 [1]: Fig. 1c, d), was observed only in *Cistoclemmys*. The shape of the iliac blade appears to be associated with the closure of the posterior plastral lobe (Bramble, 1974). We thus suspect that the most prominent difference in shell-closing mechanism exists between *Cistoclemmys* and the other geoemydine box turtles, not between *Notochelys* and the others. In the Geoemydinae, the plastral hinge seems to have actually evolved three times—in the *Notochelys-Cyclemys* clade (Stem G), *Cuora* (Stem M), and the *Pyxidea-Cistoclemmys* clade (Stem Q).

In adult females of some batagurids lacking the plastral hinge, the bony connection between the hypoplastron and carapace is replaced by a ligamentous connection. Such plastral kinesis, sometimes called sexually dimorphic plastral kinesis, is probably an adaptation for the passage of relatively large eggs which otherwise could not pass through the posterior opening between carapace and plastron (Waagen, 1984; Moll, 1985). Sexually dimorphic plastral kinesis is present in *Geoemyda japonica*, *G. spengleri* (see Yasukawa et al. [1992]), *G. silvatica* (see Moll et al. [1986]), *Heosemys grandis* (Yasukawa unpublished data), *H. spinosa* (see Moll [1985]), *Melanochelys tricarinata* (see Waagen [1984]), and *Rhinoclemmys* spp. (see Pritchard and Trebbau [1984]). Therefore, all members of the *Geoemyda* group exclusive of *Melanochelys trijuga* actually show plastral kinesis at least in adult females. Considering its sporadic emergences on the phylogenetic tree of the *Geoemyda* group, it is probable that sexually dimorphic plastral kinesis represents a primitive condition in this group.

Taxonomic notes

Based on the strong plastral buttresses in *Mauremys annamensis*, Savage (1953) revalidated the monotypic genus *Annamemys* Bourret 1939, for this species. He also suggested its close affinity to several batagurines with strong plastral buttresses, such as *Batagur*, *Callagur*, *Hardella*, and *Kachuga*. On the other hand, Iverson and McCord (1994), in the analyses of morphometric variation in the East Asian *Mauremys*, regarded *annamensis* as the closest relative of *M. mutica*, and synonymized *Annamemys* with *Mauremys* again. The present study showed that the buttress of *M. annamensis* is stronger than that of other geoemydines, but much less developed than that of the above-mentioned batagurines (see comments for characters 28 and 29). Except for the buttress, this species shared most character states with *M. mutica*, supporting Iverson and McCord's (1994) account (Table 1). Assignment of this species to the genus *Mauremys* was also supported by a recent mithochondrial DNA study, which, however, suggested the closest affinity of *M. annamensis* with *M. iversoni* rather than with *M. mutica* (Honda et al., in press).

The NJ phylogram, while supporting the *aurocapitata-pani-trifasciata-mccordi-zhoui* assemblage, showed that *Cu. amboinensis*, the type species of the genus, is highly differentiated from the other congeners. Such a relationship was also supported by the cladogram as well. However, we found no character states definitely uniting the five species. Therefore, we do not further divide this genus.

Of the currently recognized species of *Cuora* (sensu lato), *Cu. yunnanensis* from Yunnan, China, was not examined in our study. Examining all syntypes (=all available specimens since the description by Boulenger [1906]) of this rare species, Ernst (1988) demonstrated a close relationship of *Cu. yunnanensis* with *Cu. trifasciata*. McCord and Iverson (1991) also suggested a close affinity of *Cu. yunnanensis* with *Cu. trifasciata*, as well as with *Cu. pani* and *Cu. aurocapitata* on the basis of morphometric comparisons among all extant *Cuora* (sensu lato). Therefore, we tentatively retain this species in the genus *Cuora* (sensu stricto).

Geoemyda silvatica is an enigmatic species endemic to the Western Ghats of southwestern India (Das, 1991; 2001). Its generic allocation has been in dispute. Some authors assigned this species to *Geoemyda*, while others assigned it to *Heosemys* (see the *Geoemyda* subsection in "Materials and Methods"). Such a taxonomic confusion is probably attributable to the paucity of studies directly comparing specimens of *G. japonica*, *G. silvatica*, and *G. spengleri*. We examined representatives of all these species in detail, and our results strongly suggest the monophyly of the three species and the validity of the *G. japonica-G. spengleri* clade.

Of the currently recognized species of *Rhinoclemmys* (sensu lato) *R. melanosterna*, *R. diademata*, and *R. nasuta* were not examined. They were once treated as subspecies of *R. punctularia*, and then were elevated to full species (Pritchard, 1979; Pritchard and Trebbau, 1984; David, 1994). Like *R. punctularia* and *R. funerea*, all of the three species are aquatic and have a shallowly notched upper beak (Ernst and Barbour, 1989). In addition, a small foramen on the ventral maxilla, which is suggested to be a unique synapomorphy of the *R. punctularia–R. funerea* clade (35 [1]), was recognized in juvenile specimens of *R. melanosterna* and *R. diademata* (Hirayama, unpublished data). We thus tentatively retain the three species in the genus *Rhinoclemmys* (sensu stricto).

Direction of future studies on the geoemydine phylogeny

In this study, we attempted as comprehensive an analysis as ever made for the phylogeny of the highly diversified subfamily Geoemydinae by best use of morphological information.

As a result, we established a best-fitting phylogenetic hypothesis at this level (Figs. 3 and 4), and accordingly suggested some changes in the classification of this subfamily (see above). Nevertheless, our analysis failed to resolve a number of ingroup relationships. Moreover, our results, as well as our presumption of the batagurine monophyly, substantially contradict the results of recent preliminary molecular studies on the batagurid phylogeny (e.g., Sites et al., 1984; Shaffer et al., 1997; Wu et al., 1998; McCord et al., 2000; Honda et al., in press). In the future, more comprehensive phylogenetic analyses of Testudinoidea using molecular data are desirable to test and revise, when necessary, the hypothesis and classification herein proposed.

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APPENDIX

Specimens Examined.—Catalogue numbers of specimens deposited in reptile collection of Kyoto University Museum, turtles collections of Madras Crocodile Bank Trust, Biological collections of Teikyo Heisei University, and private collections of Ren Hirayama and P. C. H. Pritchard are preceded by KUZ R, MCBT, THUb, RH and PCHP, respectively. The other acronyms are those suggested by Leviton et al. (1985).

Geoemydinae: *Chelopus annulatus* (2 specimens) RH861–862; *C. rubidus* (1) RH331; *Cistoclemmys flavomarginata flavomarginata* (9) NSMT02087–02089, one unnumbered specimen of OMNH-R, KUZ R19561, 36752, RH; 53, 62–63, 430; *Ci. f. evelynae* (4) NSMT02090–02092, KUZ R36746; *Ci. galbinifrons* (7) KUZ R36745, 36757, 36763, RH736–737, 750–751; *Cuora amboinensis amboinensis* (2) KUZ R36704–36705; *Cu. a. kamaroma* (13) KUZ R19583–19584, 36763, RH34–36, 519–520, 596–600, *Cu. aurocapitata* (4) KUZ R36710, THUb13–14, RH915; *Cu. mccordi* (6) THUb21–22, RH935–936, 950, 981; *Cu. pani* (4) RH901–903, 987; *Cu. trifasciata* (5) KUZ R 36709, RH219–222; *Cu. zhoui* (4) KUZ R36706, 36711, RH868, 1001; *Cyclemys* spp. (11) KUZ R 36609, 36707–36708, RH134–139, 550–551; *Geoemyda japonica* (13) NSMT02083–02086, OMNH-R333–3334, two unnumbered specimens of OPM, KUZ R36720–36721; RH480–482; *G. silvatica* (6) FMNH52515, four unnumbered specimens of MCBT, PCHP2725; *G. spengleri* (12) two unnumbered specimens of NSMT, USNM84992, KUZ R9994, 12630, 19399–19401, THUb15, RH847, 856, 857; *Heosemys grandis* (3) KUZ R19581, 36718, RH581; *H. spinosa* (4) KUZ R36757, RH185–186, 266–267; *Mauremys annamensis* (6) KUZ R36700, RH 936–939, THUb 9; *M. caspica* (1), RH666; *M. japonica* (10) KUZ R19575–19580, 36712–36715; *M. leprosa* (2) RH453–454; *M. mutica kami* (5) KUZ R19524–19525, 19541, 19543–19545; *M. m. mutica* (6) KUZ R19509–19512, RH54, 194; *M. rivulata rivulata* (6) KUZ R36716, 36761, RH541–542, 888, 929–930; *Melanochelys trijuga* (10) KUZ R36718–36720, four unnumbered specimens of MCBT, RH238, 549, 557; *Notochelys platynota* (6) KUZ R36721–367222, RH858, 869, 961–962; *Pyxidea mouhotii* (5) KUZ R36723, RH240, 246–247, 269, 273; *Rhinoclemmys areolata* (2) RH654–656; *R. funerea* (3) 978, 985–986; *R. pulcherrima mani* (5) KUZ R36724, THUb17–19, RH835, 836; *R. punctularia* (4) 239, 248, 457, 510; *Sacalia bealei* (2) RH525–526; *S. quadriocellata* (2) KUZ R36725, RH709; *Sacalia* sp. (2: skeletal specimens not identified at the species level) RH308, 524. Btagurinae: *Batagur baska* (2) one unnumbered specimen of MCBT, RH 1002; *Callagur borneoensis* (3) KUZ R19560, RH287, THUb10; *Chinemys reevesii* (10) KUZ R 19591–19593, 36692–36698; *Geoclemys hamiltonii* (2) one unnumbered specimen of MCBT, RH920; *Hardella thurjii thurjii* (3) KUZ R 36699, 36701, THUb35; *Hieremys annandalii* (3) RH236, 587, 928; *Kachuga smithii smithii* (5) KUZ R 36759–36760, RH281, THUb49, 52, *Malayemys subtrijuga* (4) RH140–143, *Morenia petersi* (2) KUZ R 36702–36703, *Ocadia sinensis* (4) RH201–202, 340–341; *Orlitia borneensis* (1) RH871; *Siebenrockiella crassicollis* (5) KUZ R36762; RH116, 706–708.

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Abstracts in Japanese will appear in Bulletin of the Herpetological Society of Japan, Vol. 2002, No 1.

[Turtles]

1. Ecology of *Trachemys scripta* on Okinawa Island. By: Dai Hatanaka, Takeshi Sasaki, Yoshitsugu Kawashima, and Go Ogura.
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3. Distribution and population structure of the common snapping turtle, *Chelydra serpentina*, in Inbanuma Basin. By: Raita Kobayashi, Noriyuki Hashimoto, and Masami Hasegawa.
4. In which seasons do *Chinemys reevesii* and *Mauremys japonica* grow? By: Yasuhiro Kosuge and Masami Hasegawa.
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7. Female-biased mortality and it's consequence on adult sex ratio in the *Chinemys reevesii* on an island. By: Toshiaki Takenaka and Masami Hasegawa.
8. Comparison of the shell formation pattern between *Caretta caretta* and *Pelodiscus sinensis*. By: Masayoshi Tokita and Shigeru Kuratani.
9. Phylogenetic relationships and biogeography of testudinid turtles. By: Yuichirou Yasukawa, Hisashi Kimoto, and Akira Taketo.

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10. Molecular evolution of ρB-crystallin of the gecko, *Lepidodactylus lugubris*. By: Yutaka Fujii, Hisashi Kimoto, Keiko Ishikawa, Yuichirou Yasukawa, Saiko Yamashiro, Kikuko Watanabe, and Akira Taketo.
11. Ecological observations of the chameleons *Frucifer oustaleti* and *F. rhinoceratus* in a dry forest of western Madagascar. By: Masami Hasegawa, Akira Mori, and Isami Ikeuchi.
12. Phylogenetic relationships of water skinks of the genus *Tropidophorus* (Reptilia: Scincidae). By: Tsutomu Hikida, Masanao Honda, and Hidetoshi Ota.
13. Molecular evaluation of population systematics and biogeography of *Ateuchosaurus pellopleurus*. By: Masanao Honda.
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[General]

56. Amphibians and reptiles in the Red Data Book of Nagasaki Prefecture, Japan. By: Takanori Matsuo.

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For more information, please visit **www.cassovia.sk/emys**, or contact:

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